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THE PRENATAL GROWTH OF THE CAT: XII. THE WEIGHT OF THE HEART IN THE FETAL AND IN THE ADULT CAT

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(Received for publication, June 29, 1942)

The weight of the adult human heart has been studied very carefully by Greenwood and Brown (1913), Bean (1920) and others. Bach (1926) has assembled data on the weight, volume, and diameters of the male and female human heart in postnatal life. A good review of the growth of the heart in the late fetal period and in postnatal life is given by Peter, Wetzel, and Heiderich (1938). The size of the heart in the living has been studied by means of X-rays by Bardeen (1918) for the adult heart, and the size of the heart in the child has been studied by Josephi (1935), Maresh and Washburn (1938), and by Bakwin and Bakwin (1935). Keys and Friedell (1938) have studied the effects of athletic activity upon the heart size in young men from 18 to 28 years of age, and Donaldson (1935) has summarized the effects of exercise on the heart of the rat and the dog. The only weights of the heart in the cat, so far as is known, are those given by Joseph (1908) for 11 males and 15 females and by Crile and Quiring (1940) for seven males and three females. With the exception of the work on the heart of the white rat (Donaldson, 1924, and others) the human heart seems to have received most of the attention. This is due largely to the method of determination of the heart size by means of the X-rays.

MATERIALS AND METHODS

The hearts used in this study were carefully removed from 229 fetal, 35 newborn cats which had been preserved in formalin (Latimer and Aikman, 1931), and from 104 freshly killed cats (Latimer, 1936). The same methods were used in both series, except that the fetal and newborn hearts had been preserved in formalin and the adults were studied in the fresh condition. What effect the formalin may have had on these weights we do not know. Donaldson (1924) gives the changes in brain tissue after preservation in formalin, but so far as is known

there are no data on the effect of formalin preservation on the heart weight.

In all cases the great vessels were cut close to the base of the heart and the cavities opened and all blood removed. As soon as removed from the body each heart was placed in a moist chamber together with the other viscera until it was weighed in a glass-stoppered weighing bottle on an analytical balance sensitive to 0.1 milligram. The hearts of the smallest fetuses were weighed to a tenth of a milligram but the hearts of the larger fetuses, the newborn cats, and the adult cats were weighed only to the nearest milligram.

The formulae for the rectilinear curves for the fetal and also for the adult cats were all determined by the method of least squares (Snedecor, 1938), and the formulae for the curvilinear growth curves were determined by trial and error as in the preceding numbers of this series and described in a preceding paper (Latimer, 1933). The formulae used in the determination of the usual statistical constants are those given by Dunn (1929). All of the computations were carried to more decimal places than given in the tables and all were carefully checked.

I wish to express my appreciation of the careful accurate work of Mr. Dale D. Lemon, who has assisted me with the routine statistical part of this work.

Fetal and Newborn Cats

The growth of the fetal and newborn hearts is shown, plotted on body weight and on body length in Figure 1. The rectilinear curve starting at the left and with the individual cases shown as circles, represents the growth in weight of the hearts plotted on body weight. The double circles represent the individual newborn heart weights. While there is a good deal of scatter in these newborn weights yet they are fitted by the same formula as the fetal hearts. The formula for the fetal and newborn heart weights is: $Y = 0.0072X + 0.0351$, from 4 to 200 grams of body weight, with Y representing the weight of the heart in grams and X, the body weight also in grams. The average percentage deviation of the calculated from the observed averages is 9.98 per cent.

The curve starting at 30 millimeters of body length and with the individual cases shown as solid dots represents the increase in heart weight plotted on body length. The newborn hearts are shown as dots within a larger circle, and in this case they do not follow the same growth rate as do the fetal hearts, for they are distinctly above the curve which

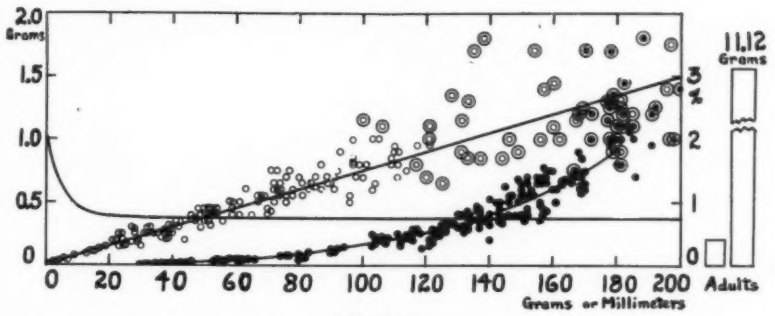

FIGURE 1

The straight line starting at the left and with the individual cases shown as circles for the fetal cats and as double circles for the newborn cats, shows the increase in heart weight plotted on body weight. The curve concave superiorly and with the individual fetal hearts shown as dots and the newborn hearts as dots enclosed by circles, shows the increase in the heart weight plotted on body length. Empirical formulae for both curves are given in the text. The lighter line without any cases represents the percentage weights of the heart plotted on body weight. The percentages are given at the right and the weights in grams at the left. The abscissae represent either weight in grams or body length in millimeters. The shorter column at the right shows the proportionate percentage weight of the adult male heart and the taller broken column, the absolute weight of the adult male heart.

has been fitted to the fetal hearts. The formula from which this curve was drawn is:

$Y = 0.00000057X^{2.74}$, from 30 to 184 millimeters of body length. Y represents the weight of the heart in grams and X, the body or nose-anus length measured in millimeters. The average percentage deviation for this formula is 8.85 per cent. This curve based on body length does not rise as high as the curve for the heart weight on body weight, but it must be remembered that this curve represents the growth of only the fetal cats while the first curve, or that based on body weight, represents the growth of the fetal and the newborn cats as well, and so it rises to a higher level. The same heart weights were used in developing both curves. The difference is in the use of the body weight or the body length as the abscissa.

The body weight seems to be a better criterion of the heart weight for these fetal and newborn cats than body length. The average percentage deviation is somewhat greater for the cases plotted on body weight but this average deviation includes the newborn as well as the fetal cats. If the average deviation for only the fetal cats plotted on body weight is found, it is 8.16 per cent, which is less than the average for the fetal hearts plotted on body length. There seems to be no de-

crease in the heart weight at birth as has been reported for man (Scammon, 1928) and for the chick (Latimer, 1928). There is no evidence of a postnatal decrease in the entire body weight of the cat (Latimer and Ibsen, 1932) as has been found in man and in the chicken. More data on the changes in the postnatal heart weight might show a decrease, for the placental circulation is lost in the cat as well as in man. In describing the growth of the human heart, Scammon (1928) says, "There is a marked postnatal decrease in growth (in both heart weight and dimensions) which may be associated with the loss of the placental bed."

The weight of the heart as a percentage of the body weight and plotted on body weight, is shown in the line without any cases. It starts at a little above two per cent, decreases rather rapidly to about 20 grams of body weight and from this point on it forms a horizontal line, with an average value of 0.76 per cent, with no appreciable change in the newborn. The shorter column at the right represents the average percentage weight of the adult heart, or 0.4 per cent. The taller broken column represents the average weight in grams of the adult male heart. These percentage weights of the heart show that it is precocious in its growth. This may further be shown by the fact that at birth the heart has attained about 11 per cent of its adult weight (male), while at birth the body weight is but 4.4 per cent of the weight of the adult male cat.

In general, the heart weight increases as an arithmetical function of the body weight in both the fetal period and for the early newborn cats. The weight of the heart increases as a geometrical function of the body length, and the formula for the fetal heart weight based on body length will not predict the weight of the heart in the early newborn cats. The heart decreases in its percentage of the body weight up to about 20 grams of body weight and then averages 0.76 per cent throughout the remainder of the fetal period and for the newborn cats as well. In the adult cat, the percentage decreases to an average of 0.4 per cent of the body weight.

Adult Cats

The average weights in grams and the coefficients of variation are given for the male and the female cats in the first two lines of Table 1. The last column shows that the absolute weights of the hearts are significantly greater in the males. The female hearts are lighter and also less variable. A comparison of the coefficients of variability of the

TABLE 1

ABSOLUTE AND PERCENTAGE WEIGHTS OF THE HEART IN THE ADULT CAT

	Average weight in grams	Coefficients of variation	Difference P. E. Diff.
Adult males	11.120 ± 0.290	27.88 ± 1.98	3.80
Adult females	9.777 ± 0.202	22.04 ± 1.46	
	Average percentage weights		
Adult males	0.394 ± 0.004	10.96 ± 0.73	1.24
Adult females	0.401 ± 0.004	10.67 ± 0.71	

heart weight and the entire body weight (Latimer, 1936) in the male cats shows that the heart is 11 per cent more variable, and in the females, the heart is 5 per cent more variable than the body weight.

The last two lines show the same statistical constants for the weights of the hearts as percentages of the body weight. There is no significant difference in the average percentage weights, but the females are slightly greater. It has been shown (Latimer, 1936) that the male cats have a statistically heavier body weight. These percentage weights are very slightly less variable in the females, but there is less sex difference than for the absolute weights. These hearts have a somewhat lower average percentage weight than the figures given by Joseph and also by Crile and Quiring, but the averages given by these workers fall within the range of my percentages. Scammon (1933) gives the percentage weight of the human heart at the close of the fetal period as 0.7 per cent, with a decrease to from 0.4 to 0.45 per cent in the adult.

The average weights and percentage weights given in Table 1 show the values for average sized cats but they do not give as accurate an idea of the heart weight for cats above and below the average weight, and to provide a method of estimating the heart weight for these larger and smaller cats, regression lines and formulae have been determined. The individual heart weights were plotted separately for the males and the females on both body weight and body length. The regression lines are all straight lines and to save space they are not represented. The individual cases have only a moderate amount of scatter. The formulae shown in Table 2 were developed by the method of least squares. The last column shows the average percentage deviation of the calculated from the observed averages for each 500 grams increase in body weight or each 25 millimeters increase in body length.

TABLE 2

EMPIRICAL FORMULAE AND AVERAGE PERCENTAGE DEVIATION OF THE CALCULATED VALUES FROM THE OBSERVED VALUES

Empirical formulae	Average percentage deviation of calculated, from observed values.
Male heart (grams) = 0.004 (body weight, grams)	2.44
Female heart (grams) = 0.00356 (body weight, grams) + 1.073	4.12
Male heart (grams) = 0.068 (body length, mm.) — 24.27	4.87
Female heart (grams) = 0.077 (body length, mm.) — 29.416	2.69

These average percentage deviations are computed without reference to sign. These formulae are valid only within the range of body weight of these cats or, from 1700 to 4700 grams, for the males and from 1500 to 3500 grams of body weight for the females. The body lengths are: 450 to 600, for the males and 450 to 570 millimeters for the females. Greenwood and Brown (1913) employed a regression formula for determination of the heart weight in man, but their formula was based on body weight and kidney weight, or on body weight, kidney weight, and the weight of the liver. They state: "We accordingly conclude that the weight of the heart may be predicted from that of the body and kidneys with an average error of some 8 per cent of the mean weight of the heart, and that the introduction of the liver does not appreciably improve the accuracy of the result."

The percentage weights of the heart of both the males and the females were plotted on body weight in the same manner as the absolute weights and smoothed curves drawn through the averages for each 500 grams increase in body weight. For the male cats there was some variation, but the line which best fitted the cases was a horizontal straight line, or the percentage weight of the heart does not change with the size of the adult male cat. The curve for the females however, started at about 0.38 per cent, rose to 0.42 per cent at its maximum at about 2700 grams of body weight, and then decreased to 0.34 per cent in the largest cats, or the female heart is relatively largest in the middle sized cats.

CORRELATIONS

The correlations between the weight of the heart and the body weight, the body length, and the weights of some of the other viscera are shown in Table 3. The average of the 20 male correlations is +0.698, and for the 21 female correlations, +0.623, or the average of the male correlations is 12 per cent higher than in the females.

TABLE 3

CORRELATIONS WITH THE HEART WEIGHT IN THE ADULT CATS

	Males	Females
Body weight	+ 0.849 ± 0.026	+ 0.868 ± 0.023
Body length	+ 0.732 ± 0.043	+ 0.796 ± 0.034
Integument	+ 0.892 ± 0.019	+ 0.826 ± 0.030
Musculature	+ 0.908 ± 0.017	+ 0.754 ± 0.042
Skeleton	+ 0.722 ± 0.046	+ 0.587 ± 0.064
Digestive tube weight	+ 0.678 ± 0.051	+ 0.561 ± 0.064
Digestive tube length	+ 0.522 ± 0.068	+ 0.524 ± 0.068
Liver	+ 0.778 ± 0.037	+ 0.621 ± 0.058
Pancreas	+ 0.581 ± 0.063	+ 0.656 ± 0.053
Lungs	+ 0.660 ± 0.053	+ 0.424 ± 0.077
Thyroid	+ 0.796 ± 0.034	+ 0.413 ± 0.078
Spleen	+ 0.645 ± 0.055	+ 0.559 ± 0.064
Suprarenals	+ 0.641 ± 0.055	+ 0.720 ± 0.045
Hypophysis	+ 0.212 ± 0.090	+ 0.522 ± 0.069
Kidneys	+ 0.912 ± 0.016	+ 0.886 ± 0.020
Bladder	+ 0.868 ± 0.023	+ 0.432 ± 0.076
Gonads	+ 0.847 ± 0.026	+ 0.526 ± 0.068
Uterus	———	+ 0.647 ± 0.057
Brain	+ 0.336 ± 0.083	+ 0.413 ± 0.077
Spinal cord	+ 0.652 ± 0.054	+ 0.737 ± 0.043
Eyeballs	+ 0.724 ± 0.044	+ 0.612 ± 0.059

The highest and likewise the lowest correlations are found in the males. There are two correlations over +0.9 in the males (kidneys and musculature) and none in the females. The organ best correlated with the heart weight in both sexes is the kidneys, and in both sexes the third in order of correlation is the integument. In the males the musculature is second from the highest, and in the females the body weight is second. The lowest correlation (hypophysis, +0.212) is found in the males. The correlations between the hypophysis and the brain in the males are both less than +0.4 and lower than any found in the females. All of the correlations are positive, although some in both sexes are so low that they are not significant.

Greenwood and Brown (1913) give correlations between the heart weight in adult men and the weight of the body, kidneys, liver, brain, and body length. All of these correlations are lower than the corresponding correlations for both the male and the female cats.

In general the weight of the heart of the cat is rather well correlated with body weight and body length and with most of the organs. These correlations average much better than for some of the other systems of the cat already reported.

SUMMARY

Empirical formulae are given by means of which the weight of the

heart may be estimated from either body weight or body length, in the fetal period, and for the adult cats. Separate formulae are given for the adult males and females, but only one formula for the fetal cats as there is no apparent sex difference in these specimens.

The absolute weight of the adult male heart is statistically greater, but the heart forms a slightly larger percentage of the body weight in the adult female. The absolute weights are more variable than the percentage weights for both sexes in the adult cat.

The heart forms a little over two per cent of the body weight at the beginning of the fetal period. This percentage drops rapidly at first and after 20 grams of body weight averages 0.76 per cent including the newborn cats. This decreases to about 0.4 per cent in the adults.

The heart is well correlated with body weight and body length and with the weights of most of the organs. The average of these correlations is 12 per cent greater in the males.

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A SURVEY OF MALPIGHIAN TUBE COLOR IN THE EYE COLOR MUTANTS OF *DROSOPHILA MELANOGASTER*

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In many kinds of experiments with wild type and mutant strains of *Drosophila melanogaster*, classification of the genotype of larvae is an essential procedure. There are, however, only three larval characters that can at present be used as reliable markers: the color of the larval mouth-parts, which is affected by certain mutations having an effect on adult body color; pigmentation of the spiracle sheath, which is affected by the ebony alleles (Brehme, 1941); and the color of the Malpighian tubes, which is related to the adult eye color. In order to increase the usefulness of the data on Malpighian tube color, the following survey has been made in which published information is brought together with a number of observations not hitherto reported.

It was observed by Beadle (1937 *a* and *b*) that the larval Malpighian tubes of certain eye color mutants differ from the bright yellow color which characterizes the wild type. He described about two dozen mutant genotypes, classifying them in four groups according to the color of the tubes: bright yellow, pale yellow, very pale yellow, and colorless. The tube color of seven other eye color mutants was described by Bridges and his co-workers (1938). These descriptions are included in Table 1. In addition, 73 mutant eye color stocks have been examined and the tube color reported here for the first time, a survey of virtually all the eye color mutations available in the United States in the year 1941. A preliminary account of this study has been made in abstract (Brehme, 1942).

Examination has been made of the stocks maintained at the laboratory of the Carnegie Institution of Washington at Cold Spring Harbor, as well as a large number of those available at other laboratories, secured through the kindness of numerous investigators. Mass matings were used, and eggs were collected by the bottle-cap method. The larvae were cultured in Petri dishes on the standard banana-agar medium, sown with live yeast suspension. Crowded cultures were dis-

TABLE 1

THE MALPIGHIAN TUBE COLOR OF THE LARVAE OF EYE COLOR MUTANTS OF *Drosophila Melanogaster*

Locus	Genetic map position	Mutant	Tube color	Classifiable
bordeaux	1- 12.5	bo	bright yellow (Beadle, 1937b)	No
bright	2- 54.3	bri	pale yellow (Beadle, 1937b)	Yes
brown	2-104.5	bw	pale yellow (Beadle, 1937b)	Yes
		bw^A, bw^D, bw^{V2}, bw^{V3}, bw^{V4}, bw^{V5}, bw^{V7}, bw^{V29l}	bright yellow	No
burnt orange	2- 57.1	buo	colorless	Yes
claret	3-100.7	ca (Beadle, 1937b), ca^2	colorless	Yes
carnation	1- 62.5	car	pale yellow (Beadle, 1937b)	Yes
cardinal	3- 75.7	cd	bright yellow (Beadle, 1937b)	No
clot	2- 16.5	cl, cl^2	pale yellow	Yes
carmine	1- 18.9	cm (Beadle, 1937b), cm^2	very pale yellow	Yes
cinnabar	2- 57.5	cn (Beadle, 1937b), cn^2 (as cn/cn^2), cn^3	pale yellow	Yes
cream—3	3- 36.5	cr-3	with w^e, colorless; without w^e, bright yellow	
cream underscored	2- 52.5	cr-u	with w^e, colorless; without w^e, bright yellow	
dark eye	2- 73	dke	close to bright yellow	No
garnet	1- 44.4	g, g^2 (Beadle, 1937b), g^3, g^4	very pale yellow	Yes
glass	3- 63.1	gl	somewhat pale (Bridges, 1938)	No
		gl^2, gl^3	bright yellow (Bridges, 1938)	No
Glazed		In(2LR)Gla	somewhat lighter than wild type	Very difficult
Henna	3- 23.0	Hn	considerably lighter than type	Difficult
		Hn^r	bright yellow (Beadle, 1937b)	No
karmoisin	3- 52.0	kar	considerably lighter than type	Difficult
		kar^2	bright yellow	No
light	2- 55.0	lt	from lt/lt mothers, colorless; from lt/+ mothers, bright yellow (Beadle, 1937a)	
		lt^3	lt/lt^3 from lt/lt mothers, colorless (Bridges, 1938)	Yes
lozenge	1- 27.7	lz, lz^{36c}	considerably lighter than type	Difficult
		lz^3, lz^{34k}, lz^{37h}, lz^g, lz^s	somewhat lighter than type, variable	No
maroon	3- 49.7	ma	pale yellow (Beadle, 1937b)	Yes
mahogany	3- 88	mah	bright yellow (Beadle, 1937b)	No
maroon-like	1- near v	ma-l	bright yellow	No

Locus	Genetic map position	Mutant	Tube color	Classifiable
Moire	3- 20	Mé	considerably lighter than type	Difficult
pink	3- 48.0	p, p^{p} (Beadle, 1937b)	colorless	Yes
		p^{Gr}	bright yellow (a few pale yellow)	No
Pale		T(2;3)P	colorless	Yes
purpleoid	2-106.4	pd	bright yellow (Beadle, 1937b)	No
Purpleoider	3- 46	Pur	bright yellow; with pd, bright yellow	No
persimmon		In(3L)pers	almost colorless	Yes
Plum		In(2LR)Pm, In(2LR)Pm^{2}	close to wild type	No
prune	1- 0.8	pn, pn^{2} (Beadle, 1937b), pn^{5}	bright yellow	No
purple	2- 54.5	pr	pale yellow (Beadle, 1937b)	Yes
		pr^{bw}	slightly lighter than type	No
Punch		T(2;3)Pu	somewhat lighter than type	Very difficult
raspberry	1- 32.8	ras, ras^{2}, ras^{3}	close to bright yellow	No
ruby	1- 7.5	rb	very pale yellow (Beadle, 1937b)	Yes
rose	3- 35.0	rs^{2}	pale yellow	Yes
rosy	3- 51	ry	considerably lighter than type	Difficult
sepia	3- 26.0	se	bright yellow (Beadle, 1937b)	No
sepiaoid	3- 64.5	sed	bright yellow (Beadle, 1937b)	No
safranin	2- 71.5	sf	pale yellow	Yes
		sf^{2}	bright yellow (Beadle, 1937b)	No
sunburst	3- 34 or 47	snb	somewhat lighter than type	No
sparkling	4-	spa	slightly lighter than type	No
scarlet	3- 44.0	st (Beadle, 1937b), st^{sp}	pale yellow	Yes
vermilion	1- 33.0	v (Beadle, 1937b), v^{2}	pale yellow	Yes
		v^{36f}	very pale yellow	Yes
white	1- 1.5	w	colorless (Beadle, 1937b)	Yes
		w^{a}, w^{a2}, w^{a3}, w^{a4}	colorless	Yes
		w^{bf}, w^{bf2}, w^{bf3}	very pale yellow (w^{bf} tested at 19° and 25° C.)	Yes
		w^{b1}	very pale yellow at 25°, darker at 19°	Yes
		w^{ch}	colorless (Bridges, 1938)	Yes
		w^{co}	pale yellow	Yes
		w^{co1}	pale yellow	Yes
		w^{e} (Beadle, 1937b), w^{e2}	colorless	Yes
		w^{h}	colorless	Yes
		w^{i}	colorless	Yes
		w^{p}	colorless	Yes
		w^{sat}	close to bright yellow	No
		w^{t}	colorless	Yes

carded, but beyond this there was no attempt to control the number of larvae in a culture. All material was maintained at 25° Centigrade unless otherwise stated in Table 1.

Larvae of the late third instar were observed on a black background under a binocular microscope, and the Malpighian tube color was examined in both the living and dissected conditions. Colorless and very pale tubes are readily classifiable from the wild type when seen through the body wall of the larva, but the pale yellow type is often distinguishable from bright yellow only when dissected. Upon dissection in Ringer's solution, tap water or distilled water, the tubes rapidly lose their yellow color, and hence must be observed immediately.

In order to make the descriptions of tube color comparable with those of Beadle (1937b), the larvae under examination were compared with wild type larvae of the Florida, Swedish-*b* or Canton-*S* stocks (representing the bright yellow classification), with a homozygous carnation stock (pale yellow) and with a homozygous carmine (very pale yellow). No controls were set up for the colorless group, since colorless tubes are unmistakable.

In classifying larval tube color, it is essential that the larvae be at the same or a comparable stage of development, since in all types of colored tubes the yellow pigment increases in amount as the larva increases in size. If care is taken that the individuals to be compared are at a similar developmental stage, classification of many genotypes is possible as early as the embryonic stage.

The results of this study are summarized in Table 1. In addition to extending the usefulness of marker loci, the list of classifiable genotypes includes two valuable chromosomal aberrations, Pale translocation and persimmon inversion.

It is evident that where alleles have a different effect on eye color, as in the cases of the white and purple loci, the Malpighian tube color of the alleles may also differ, and the amount of pigment in the tubes need not correspond to the amount of color in the eye. For example, white-blood has dark eye color but very pale yellow tubes, while the lighter eye color of white-satsuma is associated with nearly wild type tube color. In general, it may be said that alleles that have a similar eye color are also similar in Malpighian tube color, although the safranin locus may offer an exception to this.

It was found by Beadle (1937a) that one eye color mutant, light, shows a maternal inheritance of tube color, since the light offspring of

females homozygous for light have colorless tubes, while those of heterozygous females have wild type tube color. In the new material presented here, no attempt was made to detect a maternal effect. All larvae were the offspring of parents homozygous for the eye color mutant in question, except for the following cases, where lethality or other factors made this procedure inconvenient: Hn, Me, T(2;3)P, In(2LR)Gla, Pm, Pm^2, pn, $T(2;3)p^{Gr}$, pn^5, cl^2, w^{bf3}, Pu and the lz alleles.

It has been observed by Schultz and others that the white mottled mutants often show Malpighian tubes in which both white and bright yellow cells are present, although this appears not to be the case in the brown variegated series. Observations reported here on the brown variegated mutations were very kindly confirmed by Dr. H. B. Glass. The reality and significance of this difference in behavior of the two loci that show mottling will be investigated further.

No mutants other than those affecting eye color have been found to affect the color of the Malpighian tubes, although a systematic study of the body color and bristle color mutants was made in the course of another investigation (Brehme, 1941).

Summary

A survey has been made of the larval Malpighian tube color of virtually all the eye color mutants available in the United States in 1941, in order to increase the usefulness of this character in classifying larvae for experimental work.

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RADIOBIOLOGICAL STUDIES ON TISSUE CULTURES: II. THE DELAYED EFFECT OF X-RAYS ON CELL OUTGROWTH IN CULTURES OF FIBROBLASTS

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In a previous paper (Doljanski and Goldhaber) we described the effect of *x*-rays upon fibroblasts growing in vitro. Our experiments were restricted to the study of the inhibition of cell outgrowth in the irradiated culture itself. In the present paper experiments are reported in which the effect of irradiation on cell outgrowth was followed through successive passages of the irradiated colony.

A. MATERIAL AND TECHNIQUE

1. *Culture Technique*

Our experiments were carried out on standardized cultures of chicken fibroblasts derived from heart of 7-day-old embryos. The cell colonies were cultivated in hanging drops according to the usual procedure, using a mixture of fowl plasma and embryo extract as the medium. Growth curves of the cultures were constructed by the planimetric measurements of outline drawings of the surface area made every 24 hours.

2. *Radiological Technique*

Irradiations were carried out using a demountable *x*-ray tube which worked at a tension of 35 *KV* mostly on currents of 2 and 20 *MA*. The tube had a copper-anticathode and a window of aluminum foil, 30μ in thickness. Absorption analysis showed that the rays penetrating through the window foil and the 0.03 mm thick mica-coverglass of the cultures were mainly copper *K*-rays. The *x*-ray intensity at the distance of the irradiated subject was about 10,500 *r/min.* for a tube current of 2 *MA*, and about 85,000 *r/min.* for 20 *MA*.

3. *Experimental Procedure*

Fibroblast cultures were irradiated, immediately after transferring,

with the following *x*-ray doses: 2500, 5000, 12,000, 25,000 or 50,000 *r*. Subcultures were made at intervals of three days, and transferring was continued as long as the cell colonies survived. The size of the outgrowth was determined daily by planimetric measurement. Each *x*-ray dose was tested in 10 series of experiments.

B. Results

1. *Irradiation with 2500 r*

As stated in our previous communication (Doljanski and Goldhaber), an *x*-ray dose of 2500 *r* induces a just detectable inhibition of outgrowth in the majority of irradiated cultures. The irradiated cell colonies develop well and show a dense and regular growth zone. When irradiated colonies are transferred, the subcultures show good development and attain considerable size. In general, however, the growth area of the 1st and 2nd subcultures is somewhat inferior to that of the irradiated original colony. In subsequent subpassages the growth rate of the cultures increases and reverts to the original levels. The diminution of the growth rate is thus only transient. Cell strains derived from cultures irradiated with 2500 *r* showed undiminished growth intensity after 33 days of cultivation (11 passages). It seems correct to conclude, therefore, that such colonies can be cultivated indefinitely.

Representative examples of this experiment series are shown graphically in Figure 1.

2. *Irradiation with 5000 r*

As reported by us previously, irradiation with 5000 *r* causes a 40 per cent diminution in the growth rate as compared to non-irradiated controls. Nevertheless the irradiated culture develops vigorously, and forms a dense growth zone consisting mostly of cells of normal appearance.

When a culture irradiated with 5000 *r* is transferred, the subcultures show a diminished rate of outgrowth in subsequent passages. Progressive diminution of the growth rate occurs regularly in the course of passages, the zone of cell outgrowth becoming ever thinner and looser. After 3-8 passages all cell migration ceases and further cultivation of the colony becomes impossible.

The maximum number of passages through which cultivation of cell

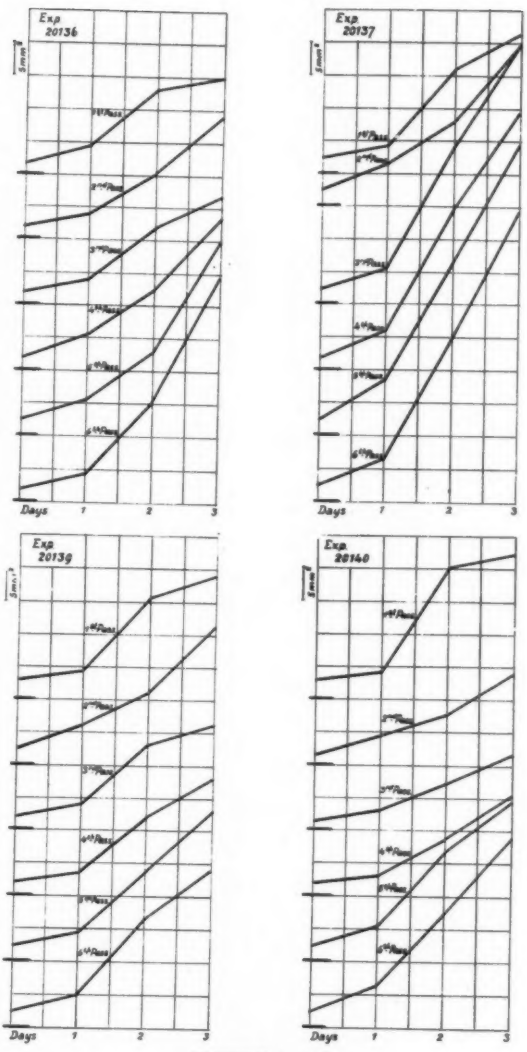

FIGURE 1, A-D

GROWTH RATE OF FIBROBLAST COLONIES DERIVED FROM FIBROBLAST CULTURES IRRADIATED WITH 2500 *r* UNITS, AND CARRIED THROUGH SUCCESSIVE PASSAGES

colonies irradiated with 5000 *r* may be continued is given in Table 1. Representative experiments of this series are plotted in Figure 2.

TABLE 1

Irradiation with 5000 *r* units

Culture No.	Passages survived
19893	7
19894	6
20141	7
20142	7
20143	7
20300	5
20809	4
20810	5
20811	4
20812	5
Mean	57

TABLE 2

Irradiation with 12,500 *r* units

Culture No.	Passages survived
23396	7
23397	4
23398	7
23522	7
23523	5
23524	6
19896	6
19897	7
19898	6
19899	4
Mean	59

TABLE 3

Irradiation with 25,000 *r* units

Culture No.	Passages survived
19850	5
19902	6
19904	8
20657	7
20661	7
20802	4
20803	4
20805	4
20806	5
20807	5
Mean	55

TABLE 4

Irradiation with 50,000 *r* units

Culture No.	Passages survived
22976	3
22977	3
22978	3
22979	4
22980	3
22981	3
22982	3
22983	3
22984	3
22985	3
Mean	31

3. *Irradiation with 12,500 and 25,000 r*

Irradiation of cultures with doses of 12,500 or 25,000 *r* causes, as has been indicated in our previous paper, roughly a 50 per cent reduction of the growth rate. Nevertheless, subcultivation of the irradiated cultures can be continued through several passages. Cultures irradiated with either of the doses lost their capacity to grow after about the same time and number of passages as those irradiated with 5000 *r*.

Tables 2 and 3 show the number of passages through which cultivation of cell colonies receiving 12,500 or 25,000 *r* may be continued. Data of representative experiments (25,000 *r*) are plotted in Figure 3.

4. *Irradiation with 50,000 r*

Irradiation with 50,000 *r* causes a 74 per cent retardation of the

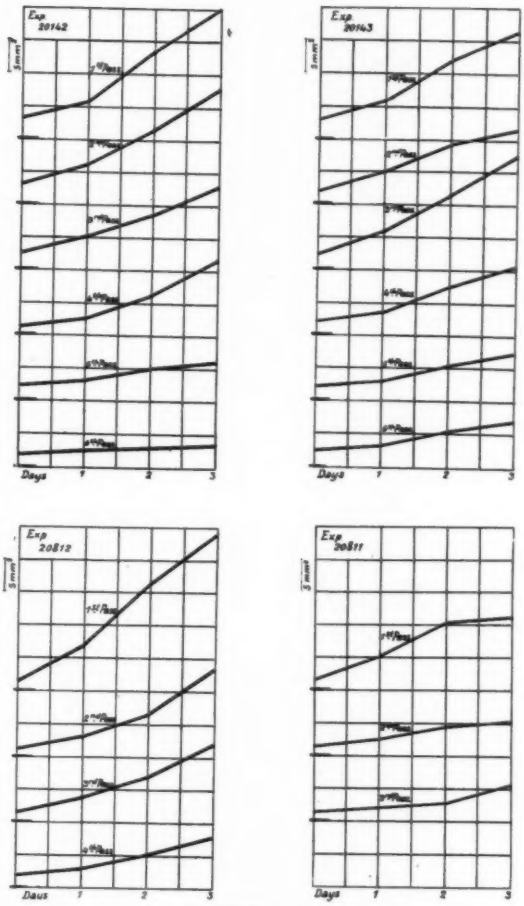

FIGURE 2, A-D

GROWTH RATE OF FIBROBLAST COLONIES DERIVED FROM FIBROBLAST CULTURES IRRADIATED WITH 5000 *r* UNITS, AND CARRIED THROUGH SUCCESSIVE PASSAGES

growth rate as compared with controls. Subcultivation is in most cases possible for only three passages. In the 4th passage cultures generally die. In one case a colony which was irradiated with 50,000 *r* could be maintained through four passages, and died in the fifth passage.

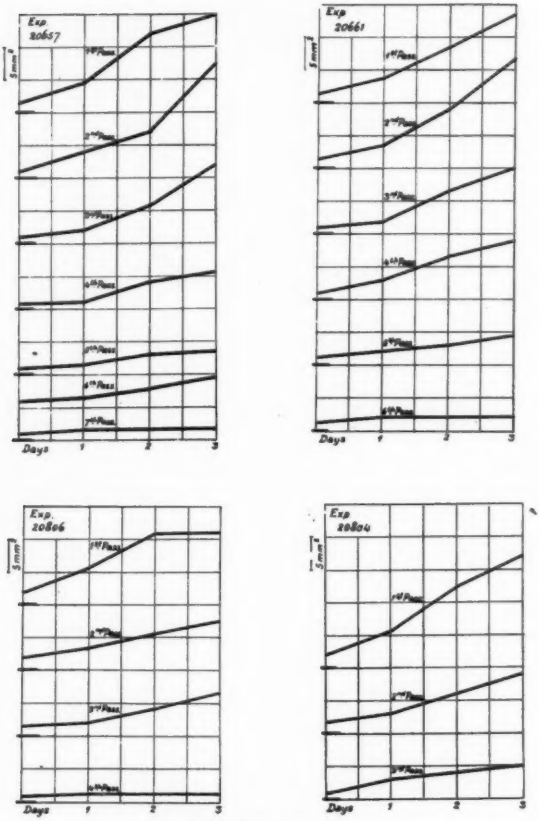

FIGURE 3, A-D

GROWTH RATE OF FIBROBLAST COLONIES DERIVED FROM FIBROBLAST CULTURES IRRADIATED WITH 25,000 *r* UNITS, AND CARRIED THROUGH SUCCESSIVE PASSAGES

Table 4 shows the number of passages through which cultivation of cell colonies irradiated with 50,000 *r* was possible.

C. DISCUSSION

Studies of the effects of radiations on cell colonies have shown that increasing doses of *x*- and radium rays cause a progressively increasing inhibition of cell outgrowth in vitro (Laser and Halberstaedter, '29;

Faber, '35; Halberstaedter and Doljanski, '37; Doljanski and Goldhaber, '42). Large doses cause an immediate and complete cessation of cell outwandering; moderate doses produce a depression of the growth rate of the irradiated cell colony. The restriction of growth rate subsequent to irradiation with weak doses becomes evident only after an interval of 24 hours.

Fischer and Baastrup ('30) and Santesson ('28) have shown that *x*-ray doses, which are without visible effect on the irradiated culture, may inhibit cell outgrowth after transferring. This delayed effect of irradiation was later systematically studied by Spear ('30). He was able to show that cultures of chick embryo choroid and sclerotic irradiated with 900-2400 milligram-hours, filtered through 0.5 mm. platinum, acting at 0.5 cm. distance, continue to grow for some time, but die after several passages. The length of the survival period depended on the amount of the irradiation. Cultures irradiated for three hours with 300 mg. radium could be transferred through an average of 10 passages after which they died. When cultures were irradiated for six hours, transferring could be carried through six passages. After irradiation for 12 hours, subcultivation through three passages was possible; after irradiation for 24 hours only one passage could be effected. As the duration of irradiation was increased, the number of possible subcultures decreased. The effect of gamma rays on the survival period was less marked with 100 mg. than with 300 mg., when the same dose was used in each case. The experiments of Spear were further confirmed and extended by Cox ('31), working with *x*-rays. Cultures irradiated with 3000 *r* were found transferrable through, in average, nine passages. At a dose of 6000 *r* the average survival was 5.5 passages, at 9000 *r* three passages, and at 12,000 *r* 1.3 passages. A 2:1 variation of intensity had no appreciable effect on the changes induced by 3000 *r*, while the same variation of intensity caused a dose of 12,000 *r* to be considerably more effective at a larger intensity than at a smaller one.

It must be concluded from the foregoing experiments that doses which are themselves insufficient to check growth in the irradiated cell colony itself cause a delayed lethal effect which only becomes manifest after a number of passages. The greater the dose applied, the smaller the number of passages through which the irradiated culture can be transferred.

Our observations confirm that *x*-ray doses, which are themselves too

low to produce immediate cessation of growth in the irradiated culture, are capable of exerting a delayed lethal effect. The dose which produced a delayed lethal effect on fibroblast cultures in vitro, was found to be 5000 *r*. Cultures irradiated with 5000 *r*, though they show a decidedly lowered growth rate, develop to a considerable size. Their subcultures manifest a progressive increase of the growth rate in successive passages until finally all emigration ceases. Total cessation of outgrowth occurs in the 3rd to the 6th passage.

Contrary to Spear and Cox, we find that even five-fold increase of the dose which produces the delayed lethal effect fails to shorten the length of the survival period. Cultures which received 12,500 or 25,000 *r* survived for the same number of passages as cultures irradiated with 5000 *r*. A ten-fold increase of the delayed lethal dose was required in order to diminish the survival period.

The observed effect of *x*-rays on the outgrowth of fibroblast cultures in vitro may be discussed as follows. The outgrowth restriction induced by irradiation with 2500 *r* is temporary and is gradually overcome in subcultures. Doses of this magnitude and even smaller doses have, as is known, a definite inhibitory effect on mitosis (Strangeways and Oakley, '23; Strangeways and Hopwood, '26; Love, '31; Lasnitzki, '40). Their delayed effect, which becomes manifest in a transient depression of the growth rate of the subcultures, is therefore obviously attributable to a reparable injury of the mitotic activity of the cell colony. The effect of doses in the range of 5000 *r*, on the other hand, is not transient but definitive. A culture which receives such a dose, although it develops well after irradiation and differs little from cultures irradiated with 2500 *r* in both size and appearance, is destined to die. Such a culture is no longer capable of unlimited growth. The cause of the striking difference in the effect on cell outgrowth of *x*-ray doses of 5000 *r* and 2500 *r* respectively is uncertain. The difference may be one of quality or one of quantity: it may be an expression of a difference in the kind of the mitotic injury caused by the two doses employed, and it may be, also, that the deciding factor is not the severity of the mitotic injury suffered by each individual cell, but the number of cells whose mitotic ability is affected in the two cases. Close comparative analysis of the course of the mitotic process in cultures irradiated with the two doses in question may clear up this point.

Cultures irradiated with doses which induce a delayed lethal effect, though they show progressive decrease of growth rate in passages,

develop well for some time. In order to comprehend the outgrowth mechanism of such cultures it should be borne in mind that the increase in the size of fibroblast cultures in vitro is brought about by a combination of two factors—cell division and migration. Cultures irradiated with doses of over 5000 *r* suffer, as we have seen, irreparable damage to their mitotic capacity. It may be assumed, therefore, that the increase in growth area of such cultures is primarily due not to cell division, but to cell outwandering.

In order to affect cell outwandering, doses of an altogether different range of magnitude must be employed. The ray susceptibility of this cell function is relatively small. Cell migration is completely suppressed according to our experiments (Doljanski and Goldhaber), only at doses 50 times greater than the dose which produces a delayed lethal effect. Cultures irradiated with 12,500 or 25,000 *r* can be subcultivated through approximately the same number of passages as a culture receiving 5000 *r*. It is only at a dose level of 50,000 *r* that the survival period of irradiated cultures is noticeably shortened. The changed response can be explained by the assumption that doses in this range decisively affect not mitosis alone, but other cell functions as well.

D. Summary

The delayed lethal effect of *x*-rays on fibroblasts cultivated through successive passages in vitro has been investigated. The effects of doses ranging from 2500 to 50,000 *r* were compared.

Irradiation with 2500 *r* causes a transient depression of the growth rate of cell colonies cultivated in vitro. A dose of 5000 *r* produces a lethal effect which sets in after 3-6 passages ("delayed lethal effect").

On increase of dosage to 12,500 and 25,000 *r* no shortening of the survival period was observed.

The duration of the survival period was only reduced after irradiation with 50,000 *r*.

A tentative explanation of the change in the response of the cell culture with variation of the dose employed is suggested.

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A KINETIC ANALYSIS OF POTASSIUM ACCUMULATION AND SODIUM EXCLUSION

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A. INTRODUCTION

An important aspect of the problem of the growth and development of tissue is the permeability of cells to substances in their environment. One of the most puzzling features of this problem stems from the experimentally established fact that the protoplasm of living and growing cells is rich in potassium and poor in sodium, despite the fact that these cells develop in an environment where sodium is far more abundant than potassium. The question naturally arises of a mechanism which would explain how developing tissue can simultaneously accumulate from its environment an ion of low concentration and successfully prevent the influx of large amounts of a relatively concentrated one.

Evidence has been accumulating from growth rate studies of both normal and carcinomatous tissue that a high growth rate is related to high potassium content. In general this problem has taken on added significance since recent research (Fenn, 1940) has tended to emphasize the physiological importance of potassium in the growth as well as the function of tissue.

It seems reasonable to suppose that the elucidation of the rôle of K and the mechanism of its accumulation will provide interesting leads for the problem of growth and development.

Many theories have been devised to explain this finding. Unfortunately, few have been proposed with any attempt at a precise formulation. Some have implicit assumptions which violate well established physical principles. Others are dependent on forces which are inadequate to accomplish the purpose for which they have been invoked. The result has been a confusion in the midst of which many questions, amenable to solution, have gone unanswered. In view of the general

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importance of the problem it was thought desirable to analyze some of its fundamental aspects from a kinetic point of view.

The active selection of one ion and the partial exclusion of another involves the motion (or lack of it) across phase boundaries or energy barriers. The kinetics of this movement can be analyzed. With the aid of a general analysis it is possible to answer the following pertinent questions:

1. What are the physical implications of postulating a 'selective' membrane?
2. Can electrostatic barriers provide a selective mechanism?
3. Can mobility differences explain the observed phenomena of potassium and sodium balance?
4. How much energy is required to concentrate potassium in the presence of sodium to the extent found in living tissue?
5. Can physical forces such as polarizabilities, London type 'dispersion' potentials, or polar interactions supply this amount?
6. Knowing the energy requirements can one postulate a reasonable direction in which to look for a mechanism?

The present paper has in general three major purposes. They are:

1. To present the application of a generalized theory of transport to the problem of potassium and sodium balance.
2. To analyze more or less in detail some of the proposed mechanisms with the aid of the general theory.
3. To attempt the formulation in terms of energy requirements and physical reasonableness certain necessary conditions which must be met by any theory if it is to effect quantitatively the kind of separation of potassium and sodium ions such as is found in living tissue.

It is advantageous to begin with a reasonably precise statement of the general problem posed for analysis; given a number of species of dissolved ions moving into and out of a cell, is it possible to postulate physically realizable assumptions concerning the structure of the system, the forces which act in it, and the mechanism of transport, such as will predict and describe the striking and uniform difference between the steady state concentrations of potassium and sodium?

There are two major methods of attack available for setting up a theory of transport of material across boundaries between phases. One is the thermodynamic, the other is the kinetic. A thermodynamic argument has important advantages and is ordinarily used wherever feasible, since it greatly simplifies the mathematical detail and makes inter-

pretation of the calculations a relatively easy task. However the application of thermodynamics to an open system not in equilibrium is at best a questionable procedure. Under the circumstances the safer course has been adopted and a kinetic approach has been resorted to despite its relatively greater mathematical complexity. A general kinetic theory of transport has been developed by Reiner (1941). A development of this theory is used to analyze the problem being considered.

B. General Theory of Transport

There are three major assumptions made. They are: (*a*) The transport of molecules or ions is governed by the customary laws of kinetic theory. (*b*) The properties of each phase vary in some continuous fashion. (*c*) Distinct phases are so defined that, at the boundary between any two of them, these properties experience either a discontinuous change or so sharp a change that it may adequately be described by a discontinuity.

In the development of a general kinetic picture of transport from these basic assumptions several successive steps are involved. The first is to describe the behavior of a population of molecules or ions within one phase in the absence of external forces or internal inequalities, allowing them to assume purely random positions and velocities. The second is the examination of the effect on this random distribution when some external force or some internal inequality is imposed. The third and final step consists of the introduction of phase boundaries, and the study of their effect on the disturbances created by the second step. The third step only need be considered in any detail, the first two being stated briefly for the sake of continuity and ease in following the argument.

1. *Step 1: Random Distribution within a Phase*

To describe a set of similar molecules statistically it suffices to state how many of them have a given velocity and a given direction, or what amounts to the same, how many are characterized by a given value of the three rectangular components of velocity. The function which gives this information is the distribution function of velocity.

For a perfect gas having uniform concentration and experiencing no external forces of a directional kind, motion must be random; i.e., any direction of motion is as likely as any other, and the distribution func-

tion should be a function only of the magnitude of the velocity. Kinetic theory shows that this function has the form

$$N(c) = \left(\frac{m}{2\pi kT}\right)^{3/2} ne^{-\frac{mc^2}{kT}} \tag{1}$$

where, $N(c)$ = number of ions per c.c. possessing velocity c
m = mass of ion in grams
k = Boltzman's constant
T = Absolute temperature
n = concentration in molecules per c.c.
c = velocity in cm. per second

This is the Maxwell-Boltzman distribution function and represents the number of ions in the population which have the velocity whose numerical value appears in the exponent. When plotted out it gives the familiar bell-shaped curve.

2. *Step 2: Imposition of Concentration Gradient within a Phase*

If now a concentration gradient is imposed on the population the velocity distribution will be disturbed and will no longer be described by the Maxwell-Boltzman function. The concentration is now not constant, but a function of spatial coördinates. For simplicity suppose it depends only on one coördinate x, and is uniform in the y and z directions. Before the imposition of the gradient the ions moved randomly, as many molecules entered any element of volume as left it, and so the Maxwell distribution was maintained. But when the concentrations in two neighboring parts are different more molecules will leave the region of higher concentration than enter it from the region of lower concentration. Thus a diffusion flow will be set up striving to equalize the concentrations. If the system is a closed one the flow will continue until this is achieved and the Maxwell distribution reestablished. However when we consider molecules of solute flowing into a cell from the outside and being chemically transformed therein, or being produced in the cell and flowing outward, it is clear that a steady state may be established. In this state there is a constant diffusion flow in the positive or negative direction, but the concentration at any place, while not the same as at other places, does not vary as time passes. It is with such stationary states of diffusion that we are concerned.

Although in this state the Maxwell distribution does not hold, if the concentration inequalities are not too great we may with sufficient accuracy assume that the distribution is not very greatly different from a Maxwellian one. Furthermore this distribution should be such as to reduce to the latter for zero concentration gradient. We assume that the new function can be expanded as a power series in u, the x component of the velocity, with coefficients depending only on x and the total velocity c. If the justifiable approximation mentioned above is made the linear term of the expansion is retained and the new distribution function takes the form

$$f = f_o + uf(c) \tag{2}$$

In the case of a concentration gradient along the x-axis, f in equation (2) is

$$f_o = \left(\frac{m}{2\pi kT}\right)^{3/2} n(x) e^{-\frac{mc^2}{kT}} \tag{3}$$

where $n(x)$ is the concentration at x and is thus the ordinary Maxwellian distribution with a variable n for each point. $F(c)$ is determined by means of the so-called integro-differential equation of Boltzman which every distribution function must satisfy. This equation is a sort of balance-sheet for the various processes which may cause a distribution to change with time. When these are added up for the case being considered $F(c)$ turns out to be

$$F(c) = -\left(\frac{L}{c}\right)\frac{\delta f_o}{\delta x} \tag{4}$$

where L is the mean free path.

The diffusion current given by the total number of ions that will cross a unit area per unit time must now be calculated. Since a statistical distribution of velocities must be considered, the net flow for each is considered separately and the total diffusion current is then calculated by summing them all up. This is given by,

$$J = \int_{-\infty}^{+\infty}\int\int \mu f \, du \, dv \, dw \, , \tag{5}$$

where u, v, and w are the x, y, and z components of the velocities.

Carrying through the integration yields

$$J = -\frac{L}{3}\sqrt{\frac{8kT}{\pi m}}\left(\frac{dn}{dx}\right) \tag{6}$$

This equation is identical with Fick's law if the diffusion coefficient is given by

$$D = \frac{L}{3}\sqrt{\frac{8kT}{\pi m}} \tag{7}$$

3. *Step 3: The Effect of Phase Boundaries on the Diffusion Current*

Now if the diffusion current is to cross a boundary between two phases, several other factors must be taken into consideration. At the boundary there is a change from one set of parameters (e.g., L, $\frac{dn}{dx}$) to another. Thus the function describing the velocity distribution in one phase may be and generally is different from that of another phase. Since the f's are now different, it becomes necessary to split the integral of equation (6) into two parts, one describing the flow in one direction across the boundary and the other the flow in the reverse direction. The net flow will be the difference between them.

Again, a molecule in crossing the boundary may undergo a change in potential energy which means that for one of the directions a potential barrier exists, Figure 1*a*. Further a more interesting and informative

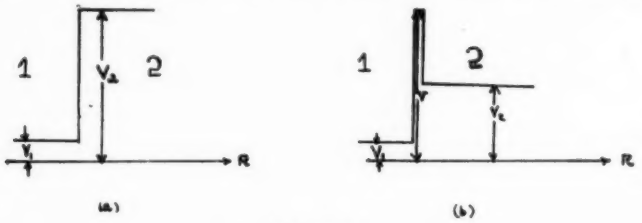

FIGURE 1
POTENTIAL ENERGY DIAGRAMS FOR TWO PHASE SYSTEMS

condition might obtain where a potential barrier exists for transport in either direction, Figure 1*b*. In this case there exists a potential barrier at the boundary superimposed on that already existing between Phase 1 and Phase 2.

Confining the discussion to the condition illustrated by 1*b* it is clear that for an ion to pass from Phase 1 to Phase 2 it must possess a mini-

mum kinetic energy equivalent to the difference between its potential in Phase 1, V, and the potential of the barrier, V. Similarly for passage in the reverse direction the ion must possess a minimum kinetic energy of $(V - V_2)$. In a population of ions obeying some statistical law of velocity distribution there will always be some fraction that will not have sufficient kinetic energy to jump the barrier. These must be omitted in calculating the diffusion current, and therefore in using equation (6) the integration is not extended over all velocities. The lower limits of integration in this case are by necessity confined to

$$\begin{aligned} \tfrac{1}{2}\, mc^2 &= V - V_1 = U_1 \\ \tfrac{1}{2}\, mc^2 &= V - V_2 = U_2 \end{aligned} \tag{9}$$

If we perform the integration using these limits and splitting our integrals as indicated above, we obtain for the diffusion current

$$J_s = \tfrac{1}{2}\sqrt{\frac{2\pi kT}{m}}\left[n_1 e^{-U_1/kT}\left(1 + \frac{U_1}{kT}\right) - n_2 e^{-U_2/kT}\left(1 + \frac{U_1}{kT}\right)\right]$$

$$- \tfrac{1}{2}\, e^{-U_1/kT}\left(1 + \frac{U_2}{kT}\right) D_1 \frac{dn_1}{dx} - \tfrac{1}{2}\, e^{-U_2/kT}\left(1 + \frac{U_2}{kT}\right) D_2 \frac{dn_2}{dx} \tag{10}$$

But by equations (6) and (7)

$$\begin{aligned} D_1 \frac{dn_1}{dx} &= J_1 \\ D_2 \frac{dn_2}{dx} &= J_2 \end{aligned} \tag{11}$$

which represent the flow at the boundaries in Phase 1 and Phase 2 respectively. Furthermore, they must be equal to one another and to J_s, the total flow, or more material would be carried to the boundary than would be carried away, resulting in an infinite accumulation there. Introducing this condition and simplifying the notation yields

$$J_s = a\,[n_1\,\phi_1 - n_2\,\phi_2] - J_s\,\phi_1 - J_s\,\phi_2 \tag{12}$$

where,

$$a = \tfrac{1}{2}\sqrt{\frac{2\pi kT}{m}}$$

$$\phi_1 = e^{-U_1/kT}\left(1 + \frac{U_1}{kT}\right) \tag{13}$$

and

$$\phi_2 = e^{-U_2/kT}\left(1 + \frac{U_2}{kT}\right)$$

Solving for J_s

$$J_s = \left[\frac{a\phi_1}{1 - \tfrac{1}{2}(\phi_1+\phi_2)}\right]n_1 - \left[\frac{a\phi_1}{1 - \tfrac{1}{2}(\phi_1+\phi_2)}\right]n_2 \tag{14}$$
$$= a_1 n_1 - a_2 n_2$$

where

$$a_1 = \left[\frac{a\phi_2}{1 - \tfrac{1}{2}(\phi_1+\phi_2)}\right]$$
$$a_2 = \left[\frac{a\phi_1}{1 - \tfrac{1}{2}(\phi_1+\phi_2)}\right] \tag{15}$$

The extension of this analysis to the case of a membrane thick enough to be treated as a distinct phase separating cytoplasm from external environment follows fairly simply. Figure 2 gives a typical potential diagram.

Quantities referred to the cytoplasm are distinguished by a subscript *1*, membrane by a subscript m, and external medium by a subscript *2*. Membrane quantities at the cytoplasm boundary have a subscript m_1, and at the external boundary, m_2. There are potential barriers at each face of the membrane. The barrier at the inner boundary has a value denoted by V and that at the outer by W. The potential differences then are defined by

$$\begin{aligned} U_1 &= V - V_1 \\ U_{m_1} &= V - V_{m_1} \\ U_{m_2} &= W - V_{m_2} \\ U_2 &= W - V_2 \end{aligned} \tag{16}$$

Thus U_1 is the net potential hill which has to be climbed by a molecule or ion in the cytoplasm in order to get across into the membrane. Simi-

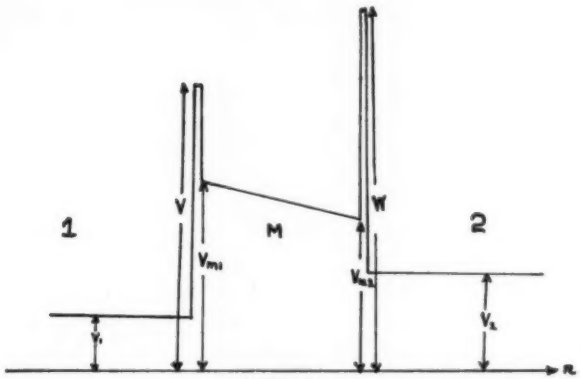

FIGURE 2

POTENTIAL ENERGY DIAGRAM, WITH SUPERIMPOSED BARRIERS AT PHASE BOUNDARIES (*1* refers to cytoplasm, *M* to membrane, and *2* to external environment)

larly for the other quantities, all of which are essentially positive or zero for the case under consideration.

With the exception that two interfaces are now involved this case is exactly the same as the one already considered. Two diffusion equations, one for each interface, must now be written. They are, for flow across the cytoplasm-membrane interface,

$$J^{1} = a_1 n_1 - a_{m_1} n_{m_1} \tag{17a}$$

and for flow across the membrane-environment interface,

$$J^{11} = a_{m_2} n_{m_2} - a_2 n_2 \tag{17b}$$

If d, the thickness of the membrane is small so that a linear concentration gradient within the membrane may be assumed, the diffusion current in the membrane can be written,

$$J_m = \frac{D_m (n_{m_1} - n_{m_2})}{d} \tag{18}$$

where D is the diffusion coefficient of the ion in the membrane. But the same approximation concerning d allows us to place

$$J^{1} = J^{11} = J_m \tag{19}$$

and any one of them may be taken as J. Eliminating n_{m_1} and n_{m_2}

between equations (18), (17a) and (17b) leads to the following relation for J_s,

$$\left(\frac{1}{a_{m_1}}+\frac{1}{a_{m_2}}+\frac{d}{D_m}\right)J_s=\left(\frac{a_1}{a_{m_1}}\right)n_1-\left(\frac{a_2}{a_{m_2}}\right)n_2 \qquad (20)$$

with

$$\left.\begin{aligned} a_1 &= \frac{a\phi_1}{[1-\tfrac{1}{2}(\phi_1+\phi_{m_1})]} \;;\; a_2 = \frac{a\phi_2}{[1-\tfrac{1}{2}(\phi_2+\phi_{m_2})]} \\ a_{m_1} &= \frac{a\phi_{m_1}}{[1-\tfrac{1}{2}(\phi_1+\phi_{m_1})]} \;;\; a_{m_2} = \frac{a\phi_{m_2}}{[1-\tfrac{1}{2}(\phi_2+\phi_{m_2})]} \end{aligned}\right\} \qquad (21)$$

where

$$\phi_1=e^{-U_1/kT}\left(1+\frac{U_1}{kT}\right) \;;\; \phi_{m_1}=e^{-U_{m1}/kT}\left(1+\frac{U_{m_1}}{kT}\right)$$

$$\phi_2=e^{-U_2/kT}\left(1+\frac{U_2}{kT}\right) \;;\; \phi_{m_2}=e^{-U_{m2}/kT}\left(1+\frac{U_{m_2}}{kT}\right)$$

These equations as formulated are applicable to all possible combinations of forces acting on the diffusing particle. Potential barriers rather than wells have been assumed in the calculation so that membrane selectivity could be discussed in the case of solutes that diffuse through the membrane into the cytoplasm and are not bound at the surface. The case of potential wells changes the signs of the potential differences and does not essentially alter the subsequent calculations. The term potential is here used in its most generalized concept encompassing chemical, electrical as well as any other form that contributes to the non-kinetic energy of the ion.

An examination of equation (20) leads inevitably to the conclusion that a description of the flow of any ion across a cell boundary involves the boundary potentials of that ion *in all the phases*. It has been inferred by some theories that an adequate approximation can be arrived at by the use of simplifying assumptions, either ignoring certain of these potentials or assigning arbitrary values to them. Whether or not such approximations are permissible must be decided by a critical analysis, on the basis of the general theory, of these proposed mechanisms which do involve such simplifying assumptions. The following

sections are concerned with these analyses. Certain theories are selected as typical for discussion.

C. Diffusion Effects

Teorell (1935) proposed a mechanism which will concentrate passive electrolytes against concentration gradients. The question that must be asked and answered is, will this mechanism, without modification, select one cation in preference to another?

According to the analysis given by Teorell, electrolytes are considered to be of two types, active and passive. Active electrolytes are supplied at a constant rate inside the cell and diffuse steadily outward. All other electrolytes are called passive. If the mobilities of the active cation and anion differ, their diffusion will set up a space charge and therefore an electrical field by which any passive electrolytes in the medium will be moved. This field will be opposed by the concentration gradient of the passive ions, and when the two influences are equal the passive electrolyte will cease to flow.

The relevant special assumptions made by Teorell in his analysis are: (*a*) no chemical reactions take place involving the passive electrolytes; (*b*) no fields are present other than those due to the ionic space charge.

The theory does not assume any distinction in structure or composition between cell, cytoplasm, and external medium. The membrane is characterized only as "supporting concentration gradients" and "containing water"; i.e., Teorell conceives it as an inert membrane of the pore type whose only effect is on mobilities.

For simplicity at first only one passive electrolyte will be considered. The extension to any number involves no special difficulties, the additional equations having precisely the same functional form. The notation is as follows; passive electrolytes will bear a subscript p, active electrolytes will bear none, cations will bear a superscript $(+)$, anions a superscript $(-)$.

Under the restriction made by Teorell concerning electrostatic fields, there exist simple relations between the constants in the expressions for the cation and anion diffusion currents. The potentials for the two have the same magnitude and opposite sign. Considering how the derivations of the expressions will be affected if we invert an energy diagram such as Figure 2 through the axis R, it is clear that one can pass from the cation to the anion expression by the transformations:

$$U_1 \rightleftharpoons U_{m_1} \quad ; \quad U_2 \rightleftharpoons U_{m_2} \tag{22}$$

which has as its consequences

$$\begin{aligned} \phi_1 &\rightleftharpoons \phi_{m_1} \quad ; \quad \phi_2 \rightleftharpoons \phi_{m_2} \\ a_1 &\rightleftharpoons a_{m_1} \quad ; \quad a_2 \rightleftharpoons a_{m_2} \end{aligned} \tag{23}$$

The active electrolyte being produced at a steady rate of q molecules per c.c. per second, then, in the steady state, the total amount produced must equal the total amount flowing out across the surface. Thus if the cell has a Volume V_o and surface area S, we can write for the active electrolyte, from equation (20):

$$J^+ = \frac{a_1 a_{m2} n^+_1 - a_2 a_{m1} n^+_2}{a_{m1} + a_{m2} + \frac{d a_{m1} a_{m2}}{D^+_m}} = \frac{q^+ Vo}{S} \tag{24}$$

$$J^- = \frac{a_2 a_{m1} n^-_1 - a_1 a_{m2} n^-_2}{a_1 + a_2 + \frac{d a_1 a_2}{D^-_m}} = \frac{q^- Vo}{S} \tag{25}$$

Note that if $q^+ = q^-$ then $J^+ = J^-$, which is Teorell's equation (11). For the passive electrolyte, the flow in the steady state is zero. Therefore,

$$\frac{a_1 a_{m2} n^+_{1p} - a_2 a_{m1} n^+_{2p}}{a_{m1} + a_{m2} + \frac{d a_{m1} a_{m2}}{D^+_{mp}}} = 0 \tag{26}$$

and

$$\frac{a_{m1} a_2 n^-_{1p} - a_{m2} a_1 n^-_{2p}}{a_1 + a_2 + \frac{d a_2 a_1}{D^-_{mp}}} = 0 \tag{27}$$

From (26 and 27), we get:

$$a_1 a_{m2} n^+_{1p} - a_2 a_{m1} n^+_{2p} = 0 \tag{28}$$

$$a_{m1} a_2 n^-_{1p} - a_{m2} a_1 n^-_{2p} = 0 \tag{29}$$

From equations (28) and (29) we can derive the following relation between the ratios of passive anions and cations:

$$\frac{n^+_{1p}}{n^+_{2p}} = \frac{n^-_{2p}}{n^-_{1p}} = \frac{a_2 a_{m1}}{a_1 a_{m2}} \tag{30}$$

which is the well known Donnan equation and was also derived in

Teorell's formulation. Carrying through the same procedure with another passive electrolyte denoted by primes we get

$$\frac{{n^+}_{1p}{}^1}{{n^+}_{2p}{}^1} = \frac{{n^-}_{2p}{}^1}{{n^-}_{1p}{}^1} = \frac{a_2{}^1 a^1{}_{m1}}{a_1{}^1 a^1{}_{m2}} \tag{31}$$

Examination of equations (30) and (31) shows that the ratios of the concentration of each passive electrolyte inside and outside is determined by the ratios of the products of the a constants. From equations (21) it is seen that these constants are functions of the potentials of the ions in the various phases. Under the restrictions of Teorell's assumptions the chemical and related potentials are the same for all passive electrolytes, leaving only the electrical to be considered. However, for univalent ions the electrical potentials are necessarily equivalent, and therefore the ratios of equations (30) and (31) are identical, when a population of sodium and potassium ions is being considered. We thus come to the conclusion that the Teorell mechanism as postulated, unaided by some supplementary process, cannot explain the preferential concentration of potassium in the presence of sodium, since it predicts equal concentration for both.

It might be noted that the argument was carried out with a specialized case (Donnan) of a more general 'diffusion effect'. However, the difference is one between immobility and relatively slow mobility of the active electrolyte anion. This reduces to a purely electrostatic difference and does not affect the reasoning, while saving a tediously long mathematical calculation, which arrives at the same conclusion.

It might be supposed that, coupled with this diffusion effect, a mobility difference between potassium and sodium ions might account for the preferential accumulation of K in the steady state. However, neither Teorell's equations, nor those describing the steady state concentrations arrived at by the general formulation, contain any dependence on the passive ion mobility. It is rather important to emphasize this non-dependence of the steady state concentrations of passive electrolytes on mobility values. The following calculation may make this clearer.

The non-stationary equation for any passive ion is obtained by setting the rate of change of its total amount equal to the total rate of flow across the boundary. For instance,

$$-\frac{d{n^+}_{1p}}{dt} = A{n^+}_{1p} - B{n^+}_{2p} \tag{32}$$

Treating $n_2{}^+{}_p$ as constant, as assumed by Teorell, we can solve for $n^+{}_{1p}$;

$$An^+{}_{1p} - Bn^+{}_{2p} = Ke^{-At} \tag{33}$$

where K is a constant of integration. From equation (26) we can see that the coefficients A and B are of the form:

$$A = \frac{A^1}{f(D^+{}_{mp})} \tag{34}$$

$$B = \frac{B^1}{f(D^+{}_{mp})}$$

where A^1 and B^1 are independent of D_{mp}. Thus (33) takes the form,

$$A^1n^+{}_{1p} - B^1n^+{}_{2p} = Kf(D^+{}_{mp})e^{-\frac{A^1}{f(D^+{}_{mp})}t} \tag{35}$$

The time-dependent term decreases steadily towards zero, at a rate determined by $f(D_{m^+p})$. In the stationary state it is zero, and (35) reduces to:

$$A^1n^+{}_{1p} - B^1n^+{}_{2p} = 0 \tag{36}$$

which is seen to be the same as equation (28) and is independent of D_{m^+p}.

Thus a distinction in terms of mobilities will affect only the rate of approach to the steady state, but not the steady state values themselves. It must be mentioned here however that it is perfectly conceivable that living tissue is neither in true equilibrium nor in the steady state, in which case the time-dependent term of equation (35) might become determining. If this were the case mobility relations might very well determine the concentration of ions found in cells. This however must remain an academic question until the theory of time-dependent states is developed and an experimental approach to the problem of distinguishing such states is devised.

One other pertinent remark may be made here. The steady state for some variable of a system is defined by imposing the condition that the variable shall not be a function of time. In the present case for a 'passive' ion this was accomplished by setting the net flow of this ion equal to zero. On the other hand, if one were dealing with the flow of a substance which is chemically transformed inside the cell by a metabolic process the steady state would be reached when the net flow

into the cell became equal to the rate of transformation. No such simple solution is possible in the case of an element. The usual chemical analysis of tissues does not distinguish between, e.g., potassium bound to some indiffusible anion and potassium free to move under a potential gradient. If by some metabolic process potassium were bound, there would be a net flow inward as long as this metabolic process continued. Under these conditions from data obtained by the usual chemical analysis one would conclude that the 'concentration' of potassium is continuously increasing. Properly speaking it is the 'amount per dry or wet weight' that is increasing. The term concentration has the unfortunate implication of a solute free to move in a solvent. It is clear, however, that the amount per wet weight could be made time independent in the case of a constant net inflow if simultaneously an adequate amount of water went in. This is discussed more fully in a later section.

D. Section 4: Calculation of Energy Requirements for *K* Accumulation

Before proceeding to the analysis of other theories it is necessary to perform a calculation which will yield the order of magnitude of the energy required to concentrate potassium to the extent found in cells from a mixed population of *K* and *Na* ions. This figure will enable us to make a decision as to the adequacy of certain possible physical mechanisms which one might be tempted to adopt as explanations of the observed phenomena.

The minimum energy necessary to arrive at a given state is independent of the mechanism by which it is reached. It is therefore possible to save a great deal of mathematical detail by simplifying the picture which is considered.

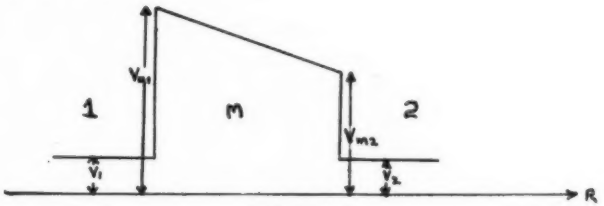

FIGURE 3
Potential Energy Diagram
(*1* refers to cytoplasm, *M* to membrane, and *2* to external environment)

Consider a membrane whose potentials for a given electrolyte with respect to cytoplasm and exterior are V_{m_1} and V_{m_2} respectively. We assume that no superimposed potential barriers exist at the two interfaces (as in Figure 2) and that $V_1 = V_2$. The potential diagram is given in Figure 3.

For an electrolyte which is not 'active' in the sense of Teorell's formulation we may set the diffusion flux equal to zero in the steady state. Using equations (20) and (21) of the general theory we get the following expression for the concentration ratio of inside to outside for one electrolyte, say K,

$$\left[\frac{n_1}{n_2}\right]_K = e^{-\frac{(V_{m_2}-V_{m_1})K}{kT}} \left[\frac{1+\frac{V_{m_2}}{kT}}{1+\frac{V_{m_1}}{kT}}\right]_K \tag{37}$$

Notice that from equation (37) we can deduce that under the conditions of its derivation a rectangular potential barrier cannot lead to concentration. This is true because such a barrier implies that V_{m_1} is equal to V_{m_2}. As a consequence the right-hand side of equation (37) reduces to unity and the inside concentration is equal to the outside concentration.

An equation similar to (37) may be written for Na:

$$\left[\frac{n_1}{n_2}\right]_{Na} = e^{-\frac{(V_{m_2}-V_{m_1})Na}{kT}} \left[\frac{1+\frac{V_{m_1}}{kT}}{1+\frac{V_{m_2}}{kT}}\right]_{Na} \tag{38}$$

To estimate the potentials for concentration and selection of potassium from a mixture of K and Na ions we combine equations (37) and (38) to get

$$\left[\frac{n_1}{n_2}\right]_K \left[\frac{n_2}{n_1}\right]_{Na} = e^{\frac{[(V_{m_1}-V_{m_2})_K-(V_{m_1}-V_{m_2})_{Na}]}{kT}} \left[\frac{\left(1+\frac{V_{m_2}}{kT}\right)_K \left(1+\frac{V_{m_1}}{kT}\right)_K}{\left(1+\frac{V_{m_2}}{kT}\right)_{Na} \left(1+\frac{V_{m_1}}{kT}\right)_{Na}}\right] \tag{39}$$

To arrive at the numerical value of the left-hand side of equation (39) the data from Fenn (1936), Table 1, for frog muscle suspended in Ringer's solution is substituted for the internal and external concentration ratios of the two ions. The value turns out to be 319. In evaluating the right-hand side of equation (39), since only orders of magnitude are desired, the exponential term only need be considered. In this way it is found that $[(V_{m_1} - V_{m_2})_k - (V_{m_1} - V_{m_2})_{Na}]$ must be roughly 5.6 times kT in magnitude or 2.3 x 10^{-13} ergs. This then represents the energy which must be supplied to selectively concentrate potassium to the extent found in frog muscle. Any mechanism attempting to explain the above finding must meet this as a necessary condition if it is to accomplish the purpose for which it was devised.

E. Section 5: Membrane Theories

In the discussion of selective membrane theories it is necessary to rule out those that involve the total exclusion of sodium by some membrane impermeability as, e.g., in the mechanism proposed by Conway and Boyle (1941). While such a postulate may be desirable on the grounds of 'simplicity' it is quite untenable in the light of the experimental data on electrolyte balance.

Recently, Heppel (1940), in living animals, and Steinbach (1940a), in resting isolated muscle, have shown that *Na* penetrates cells easily to replace potassium. As a matter of fact calculations from Heppel's data obtained with radioactive isotopes indicate that sodium penetrates four to eight times as rapidly as potassium, (Dean, 1940b). It is thus necessary to confine the analysis to such theories as will permit sodium penetration and exchange for potassium.

The feature which all selective membrane theories have more or less in common is the assumption that it is possible to account for the facts of electrolyte balance exclusively or almost exclusively in terms of special properties of the membrane itself. The usual selective membrane theory assumes that the membrane is 'critical' in determining which ion of a mixed population can get into a cell and stay there. This is presumably accomplished by means of a potential barrier or distribution of potential in the membrane or, what is equivalent, in the form of some assumption about solubility or polarization in the membrane.

It is important to emphasize that a potential barrier in one phase of a three phase system, no matter what its nature, cannot of itself determine the electrolyte distribution in all three phases. The passage

of an ion into the cell depends not merely on the height of the barrier, but on the difference between the potential of the barrier and that which exists in the external environment at the membrane boundary. Whether the ion, once having passed into the cell, will stay there, again depends on the potential at the cytoplasm-membrane boundary and its distribution in the cytoplasm, as well as on the height of the barrier. An examination of the simple case illustrated in Figure 3 makes this even clearer. If the potential of the external medium is raised until it is higher than the potential barrier in the membrane, obviously the barrier will no longer be 'critical'. It will no longer be a barrier. Furthermore, if the cytoplasmic potential is raised sufficiently, the membrane barrier will again cease to be critical.

All this is a physical restatement of a fact that was immediately deducible from equation (20) of the general theory: i.e., that description of the flow of ions across cell membranes involves the potentials in all phases. It is physically meaningless to consider one phase as 'critical' if by that is meant the others may be ignored. It is thus seen that postulating the existence of a 'critical' or 'determining' potential barrier in the membrane commits such a theory to fix the potential values in the cytoplasm and the external medium relative to the membrane. The necessity for such an auxiliary assumption raises the obvious difficulty for selective membrane theories that they involve freezing a labile system such as cytoplasm to a relatively static membrane. Disregarding this difficulty for a moment, it is proper to ask what sort of physical condition can be assumed in the membrane which will set up the necessary potential distribution.

It might be supposed that some electrokinetic property of the membrane, such as ϵ or ζ potentials at the membrane surface, could be depended upon to differentiate between potassium and sodium ions. However, such an assumption involving simple electrostatic barriers differs in no essential detail from the Teorell 'diffusion effect'. The only point of difference consists in the substitution of a static for a diffusion potential. It can no more make a distinction between equally charged ions than the diffusion potential can, since it also reduces to a question of the effect of such a barrier on mobilities, which disappear from the relation describing the steady state.

It might be argued that there could exist electrical forces or some combination of them dependent upon and residing in the membrane which could account for the observations. However, in the light of

current physical knowledge it is by no means impossible to specify the various plausible types of these forces and the contributions which they would make. These can then be compared with the value found in Section 3, for the energy necessary to accomplish the observed selective concentration of potassium. The assumptions leading to the calculations of Section 3 were particularly chosen to facilitate the discussion of such membrane theories. It is to be noted that with the cytoplasmic and external potentials equal, and in the absence of superimposed barriers which would have contributions from the cytoplasm and the external environment, the potential barrier is localized in the membrane.

Sodium and potassium have the same charge, they differ slightly in mass and radius, and both are ions of the rare gas type. They differ significantly in one respect, their polarizability; that of potassium being about four times that of sodium. There may therefore be a potential due to the interaction of charges in the membrane with the induced dipole of the ion.

To derive an expression for this potential we note that the potential energy of an induced dipole is of the form

$$\tfrac{1}{2} pF^2 \tag{40}$$

where p is the polarizability and F is the strength of the polarizing field. In this case the field is due to electrostatic charges, each ion contributing

$$\frac{e^2}{r^2} \tag{41}$$

where e is the charge and r the distance from charge to polarized molecule. We multiply by the concentration of the charges, N, in number per cubic centimeter, and integrate with respect to r over the volume of the membrane. Since F^2 varies as $1/r^4$, the potential drops off rapidly with distance. We can therefore make the upper limit infinity in the integration, and the lower limit, d, the minimum distance between the center of the inducing charge and the center of the polarized molecule. Thus we obtain,

$$U = \tfrac{1}{2}\, pNe^2 \int_a^\infty \frac{4\pi r^2}{r^4}\, dr = -\frac{2\pi p\, Ne^2}{d} \tag{42}$$

Polarizabilities are of the order 10^{-24} and d can be taken at about 10^{-7} cm.; with a generous allowance, the probable upper limit for N

may be put at 10^{+21}. Substituting these values in equation (42) it is found that the upper limit for the potentials is of the order 10^{-17} ergs. This when compared with the 10^{-13} ergs found necessary (Section 4) is about ten thousand times too small.

This is the strongest known physical force with which one could hope to devise a selective mechanism in the membrane for potassium in the presence of sodium. The interaction of polar groups of the membrane is even weaker, while a calculation of the London type of 'dispersion' potential is still less encouraging.

Selective membrane theories are thus forced to specify some as yet unknown physical force or combination of them. Such a postulate while not satisfying, might be desirable if it led to a simple theory of few assumptions. However, it is seen from the discussion that the 'simplicity' thus obtained is only apparent, for it involves implicit assumptions concerning the potential distributions in cytoplasm, membrane, and external medium. In addition, some of the relations between the distributions necessary to the theory are difficult to justify biologically.

F. Section 6: Chemical Potentials

Some authors have postulated different partition coefficients for *Na* and *K* in the various phases. These coefficients are exponential functions of the difference of potentials that the ions possess in the separate phases. Thus the general remarks concerning potential distribution in all three phases apply to these coefficients as well.

In general it is possible to divide the potentials of an ion into 'electrical' and 'chemical' parts as is done by Guggenheim (1929). Usually permeability theorists do not specify which part of the potential is responsible for the assumed difference in the partition coefficients of sodium and potassium. Meyer and Sievers (1936) in a formulation very similar to, though more restricted than Teorell's, assumed a solubility difference, without detailing any further the origin of this difference. It was this assumption that enabled them to make a distinction between *K* and *Na* that otherwise would not have been inherent in their theory. The same procedure underlies Dean's (1941) modification of the Conway and Boyle theory. It is seen from his equation (11) that his essential assumption involves different partition coefficients for the two ions. A pump of 'unknown mechanism' which acts with different effectiveness on *Na* and *K* ions is however invoked to hide this relatively simple postulate.

The analysis of the non-chemical part of the potential in previous sections has not yielded energies of the required order of magnitude. For this reason the present discussion of partition coefficients is confined to 'chemical' potentials. From an energetics point of view chemical reactions could easily fulfill the necessary condition. The value obtained in Section 4 for the energy necessary to concentrate K in the presence of Na when calculated on a molar basis comes to about 10,000 calories per mole. This is well within the range of many chemical reactions.

That relatively simple chemical systems can effectively distinguish between K and Na has been amply demonstrated by the experiments of many workers. Shedlovsky and Uhlig (1934) measured the partition coefficients of K and Na—guaiacolates between water and guaiacole and found about a four-fold difference between them. Ousterhout, Kamerling, and Stanley (1933), in their model experiments, found preferential accumulation of potassium. In these experiments a non-aqueous guiacolate layer separated by two water phases in one of which carbon dioxide was bubbled to convert the cations to the carbonates which were insoluble in the non-aqueous layer. It is of some interest to point out that the conversion to the carbonate was a necessary factor in securing preferential concentration of potassium. In an experiment where this condition was omitted the concentrations arrived at 'inside' equalled those 'outside' for both ions. This could have been predicted from equations (37), and (38). For under the conditions of this experiment the inside potential equalled that of the outside with a simple rectangular potential well interposed between. This necessarily leads to a ratio of unity of the inside to outside concentrations for both ions. The bubbling of carbon dioxide, however, depressed the potential of the interior for both Na and K in combination with the carbonate so that the well became a barrier which neither could jump.

Longsworth (1935) developed the theory of these experiments by assuming different partition coefficients for the sodium and potassium guaicolate salts in the non-aqueous layer. The solution of the resulting flow equations yielded a description of the time course of the phenomenon. It is to be noted that his final equations contain the diffusion coefficients, in contradistinction to ours. The reason for this difference lies in the fact that his condition for the steady state involved setting the rate of change of the salt concentration equal to zero rather

than the net flow as was done in the present analysis. This resulted in a positive net flow inward of salt and an amount of water sufficient to maintain the concentration at the steady state value. A condition of this nature necessarily restricts the applicability of that analysis to systems which are continually taking up water.

Another relatively simple chemical system which can distinguish between potassium and sodium was found by MacCallum (1929). He showed that a sand column in contact with an aqueous solution of these two ions selectively picked up potassium. This, like similar exchanges in zeolites, is explainable on the basis of the 'fit' of the preferred ion in the lattice structure of the solid phase.

An important contribution to the problem was recently made by Mullens (1942) who reported that myosin will selectively accumulate potassium from solutions containing both sodium and potassium.

These experiments indicate the existence of an important and specific difference between the chemical potentials of Na and K with respect to various solid and liquid phases. The establishment of this difference is of great importance if the mechanism for potassium accumulation and sodium exclusion is to be sought in the chemical potentials of these ions. This is all the more impressive since, from an energetics point of view, the present analysis indicates that a chemical mechanism would satisfy the necessary requirements.

Summary

The application of a general theory of transport has been made to the problem of potassium and sodium balance. It has been shown that:

1. Mobility differences cannot yield a mechanism for selection of one ion in preference to another by systems in the steady state.
2. Electrostatic barriers similarly cannot provide a selective mechanism.
3. Selective membrane theories in general make implicit assumptions about potential distribution in the cytoplasm as compared with the membrane which do not seem plausible and in any case would require separate justification.
4. Calculations are made which indicate that to concentrate potassium in the presence of sodium to the extent found in living tissues requires an energy supply of the order of 10,000 calories per mole.
5. Some physical forces which might conceivably be used by membrane theories were found to be inadequate.

6. It is concluded on the basis of energy requirements that chemical as distinguished from electrical forces would provide a more fruitful approach to the problem of potassium and sodium balance.

ACKNOWLEDGMENT

The authors cannot express too strongly their great indebtedness to Professor H. B. Steinbach for his invaluable advice and encouragement during both the investigation and the preparation of the manuscript. It was he who first pointed out the desirability of an analysis of this kind, and whatever is of biological value in it is due largely to his efforts.

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RELATION OF AN ESSENTIAL MICRO-ELEMENT TO SEED PRODUCTION IN PEAS

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Zinc, copper, and other micro-elements are now known to play important rôles in the growth of plants. Recent researches afford information on plant nutrition which measurably extends the older list of essential elements.

Among other reports recently published, Reed and Beck (1939) have demonstrated that zinc deficiency of the soil may depress the formation of cobs and seeds of corn (*Zea mays*) relatively more than of stalks, leaves, and husks. The corn plants grew at approximately the same rates in all cultures, whether or not zinc had been added, until nearly half the total growth had been made, due no doubt to the amount of zinc contained in the seeds plus that obtainable from the soil and other sources. During the latter half of the growth period, however, the elongation of plants where no zinc was added lagged behind the others. The curtailment in production of cobs and kernels due to zinc deficiency was greater in the third, than in either the first or second crop of corn grown in those particular lots of soil.

The growth and reproduction of peas and beans in nutrient solutions also have demonstrated (Reed, 1942) that a suitable zinc supply is essential for both processes. The plants produced seeds only when the zinc concentrations of the nutrient exceeded .02 parts per million, although they produced pods and vegetative growth at lower concentrations. There was an apparent threshold concentration of zinc, below which the plants produced only small seedless pods. Concentrations of .1 and .2 parts per million zinc resulted in the production of more and better filled seeds. The majority of the pea seeds in all cultures were viable, as shown by the percentages of germinations.

The results presented in this article throw additional light on the

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influence of zinc salts upon growth and seed production in Dwarf Telephone peas (*Pisum sativum L*).

The methods of cultivation were essentially those used in other physiological experiments, except that in all cases the solutions were artificially aerated. The plants were grown to maturity in nutrient solutions prepared by the technique described by Stout and Arnon (1939) of this laboratory. Each of the 48 culture units consisted of three plants in a two-liter Pyrex glass beaker provided with a specially constructed plaster-of-paris cover which kept the contamination of the nutrient solution at a minimum. The zinc was supplied as zinc sulfate. A few cc. of an iron sulfate solution were added twice weekly to each of the 48 beakers and the pH of the culture solutions was maintained between 4.6 and 6.0. Seven concentrations of zinc ranging from .005 to .5 parts per million were added, as shown in the third column of Table 1, when the experiment was started on April 3. The

TABLE 1

RELATION OF ZINC CONCENTRATION TO GROWTH AND SEED PRODUCTION OF DWARF TELEPHONE PEAS IN NUTRIENT SOLUTIONS

(April 3 to July 14, 1941)

Cultures	Number of plants	Zinc conc. (ppm.)	Aver. dry weight when harvested (grams) Tops	Pods	Roots	Seeds
1-6	18	0.0	3.16	0.	1.21	0.
7-12	18	.005	8.40	.05	2.75	0.
13-18	18	.01	12.12	.92	5.32	.26
19-24	18	.02	19.38	2.72	8.07	.85
25-30	15	.05	24.92	7.80	9.16	12.38
31-36	12	.1	25.78	9.58	8.53	21.27
37-42	15	.2	19.20	6.00	6.65	14.85
43-48	15	.2*	21.38	5.66	6.42	21.14

*Subsequently increased to .5 ppm.

zinc concentration in Cultures 43-48 was raised to .3 ppm. on May 15 and to .5 ppm. on May 29. Each culture received additions of KNO_3 equal to the original amount on May 16 and 27. The culture solutions in all beakers were replaced with fresh solutions of the initial composition (Table 1) on June 7 and 18, since the developing plants have reduced the amounts of the nutrient salts far below the initial amounts. The result of these replacements increased the amount of potassium nitrate five-fold and tripled the original content of zinc and other salts; however, the concentrations present at any given time were not supra-optimal.

A sample of the seeds planted contained from 8 to 9 gammas of zinc per seed. After the young seedlings had been installed in the covers, the cotyledons were removed to diminish the initial supply of zinc. The seedlings showed the effect of this amputation and did not thrive for some days thereafter, but 18 days later the tops began to grow and the plants which received sufficient zinc looked normal. All plants apparently suffered initially from an insufficient supply of carbohydrates, but after three weeks, their tops and roots were growing well. Roots in zinc-deficient cultures usually grow well, though the tops may be greatly dwarfed by the lack of that micro-element.

The pea seedlings had five to seven leaves at the end of the fourth week in all the solution cultures. The deficiency of zinc was indicated about the end of the fifth week by paleness of the younger and death of the older leaves. The tops of the plants in solutions receiving less than .01 ppm. zinc ceased growing before they were seven weeks old, although the roots were in good condition. When the cultures were seven weeks old, most of the plants in the minus-zinc solutions were dead and those receiving .005 and .01 ppm. had begun to die. Plants in solutions receiving .02 ppm. zinc and higher concentrations were beginning to form flowers. On the same date the plants in four cultures showed necrotic spots on their leaves, and unhealthy roots, the causes for which were not ascertainable. Yields of these four cultures were discarded in making up the final records. It was not possible to get accurate figures on green weights when the plants were harvested July 14, since some of the plants had been dead for a time; consequently the discussion rests on the results obtained from the dry weights of the different parts of the plants. Pollen grains from plants which received .05 and .1 ppm. zinc appeared normal in size and content when examined microscopically on June 7, and their vacuoles absorbed neutral red. Pollen from plants in lower concentrations was not available for testing at that time.

The relations between the growth of pea plants and the concentration of zinc in the nutrient solutions are shown by the data in Table 1. Without the addition of zinc the production of plant material was insignificant. The weight of tops and roots were greater in concentrations of .05, .1, and .5 parts per million zinc. The diagrams in Figure 1 show graphically the relative weights of tops, roots, pods, and seeds obtained in four selected sets of cultures.

Having seen that an extremely small amount of zinc may produce a

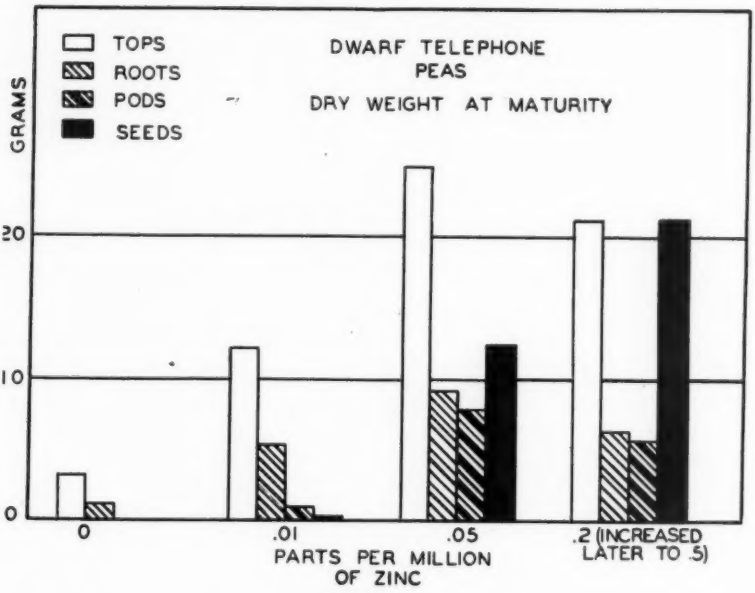

FIGURE 1

GRAPHIC REPRESENTATIONS OF THE EFFECTS OF FOUR CARDINAL CONCENTRATIONS OF ZINC ON THE PRODUCTION OF TOPS, ROOTS, PODS, AND SEEDS OF DWARF TELEPHONE PEAS GROWN IN NUTRIENT SOLUTIONS

pronounced effect upon growth and development, it is highly important to analyze the variance and thereby to estimate the probabilities that the differences between treatments were significant. Caution in draw-

TABLE 2

ANALYSIS OF VARIANCE BETWEEN TREATMENTS WITH THE CONCENTRATIONS OF ZINC TABULATED IN TABLE 1

Item	Degrees of freedom	Mean square between treatments[3]	*F*	Value of *F* required for probabilities of 99:1
Tops	7	352.83	23.60	3.20
Roots	7	43.70	12.17	3.20
Pods[1]	5	52.08	14.08	3.86
Seeds[2]	4	370.30	7.73	4.43

[1]Based upon cultures which received .01 ppm. and more of zinc.
[2]Based upon cultures which received .02 ppm. and more of zinc.
[3]These values were, in each case, calculated from separate data and each had its own error variance used in calculating the various *F* values.

ing conclusions is nowhere more necessary than in experiments like this. Table 2 contains the results of the statistical analysis of the variance between the results observed when the heretofore mentioned concentrations of zinc were supplied. F, the ratio of the variances between treatments and within treatments, is highly significant for all four items.

Pea plants which grew in a concentration of .02 ppm. zinc produced good tops and roots. Their pods, however, were small and most of them were seedless. It is highly probable that the plants contained less protein nitrogen than those which received more zinc (Bean, 1941) and could therefore supply only a limited quantity for the development of seeds, indicated by the fact that only one well-filled seed was produced (Table 3). In the concentration of .05 ppm. zinc the plants

TABLE 3

CLASSIFICATION OF SEEDS PRODUCED IN CULTURES CONTAINING VARYING AMOUNTS OF ZINC

Cultures	Zinc concentration ppm.	Number of seeds Classes* A	B	C	D	E
19-24	.02	3	1	3	1	1
25-30	.05	17	9	40	90	49
31-36	.1	26	14	45	58	137
37-42	.2	23	10	20	25	136
43-49	.2**	0	8	0	67	196

*A. Small imperfect seeds, brown to green.
B. Imperfect, shrivelled seeds, epicarps green.
C. Seeds better filled than A or B, but shrivelled.
D. Seeds smaller than the type, shrivelled, greenish epicarps.
E. Typical seeds, well filled, epicarps greenish white.
**Subsequently increased to .5 ppm.

not only produced larger tops and roots, but there was a great increase in the weights of pods and seeds (Figure 1). It seems logical to conclude that the step from .02 to .05 ppm. had crossed a definite threshold beyond which, under those cultural conditions, seeds could be produced. The differences between tops, roots, and pods of plants in .05 and .1 ppm. are not statistically significant, but there was a measurable increase in the weight and size of the seeds produced. The difference is so great that it can be referred to some sort of an auto catalytic agent which was set in operation by a trigger-action.

The contrast is still more apparent from an inspection of the results obtained from the addition of .2 ppm. (increased later to .5 ppm.) as shown in Figure 1. The weights of tops, pods, and roots were slightly

less, though the differences are statistically insignificant. The weight of seeds, on the contrary, was significantly much greater than in the cultures whose zinc concentration was .05 ppm. Although a ten-fold increase (from .05 to .5) had little effect on the production of pods, it produced a 70 per cent increase in the quantity of peas in the pods. Furthermore it must be noted that the effects began to be apparent in the plants when they were no more than seven or eight weeks old. This result is fundamentally important for understanding many of the phenomena of growth and fecundity and is worth careful consideration in connection with many problems of agricultural crop production. Cultures 37-42 which received .2 ppm. gave anomalous results due possibly to the necrotic spots on the leaves already mentioned. At any rate, the quantity of seeds produced was less than one would logically expect. Cultures 43-48 which initially received .2 parts per million with subsequent additions amounting to a total of .5 parts per million, appeared normal in every way and the yields are to be regarded with confidence.

In addition to the data presented above, there were other results of the varying amounts of zinc added which have some important biological implications. A casual inspection of the various lots of seeds revealed differences in their stages of development, and their careful classification elucidated some matters which the previous estimations had left unexplained. Figure 2 shows the classes of seeds produced in Cultures 25-30 supplied with .05 ppm. zinc, which as previously said, seemed to be just above the threshold value for seed formation, though less than the optimum. The five classes labeled *A* to *E* represent a somewhat arbitrary classification. Seeds in Class *A* were practically nothing but shriveled integuments, those in *B* were only slightly better developed; probably both classes of seeds developed from unfertilized ovules. Seeds of Class *C* were small, shriveled, greenish and had tightly adhering seed coats. Seeds of Class *D* were larger, but were not completely filled. Seeds of Class *E* were well-filled normal seeds which had good color, shape, and size. Table 3 shows the distribution of seeds of each class in the five series of cultures which actually produced seeds. The 18 plants in Cultures 19-24 produced a total of nine seeds, only one of which could be considered a mature seed. The next series of cultures, 25-30, although containing only 15 plants, produced 205 seeds, of which ¼ were typical and well-filled, and a little less than half were fairly good (Figure 2). The 12 cultures (31-36) which re-

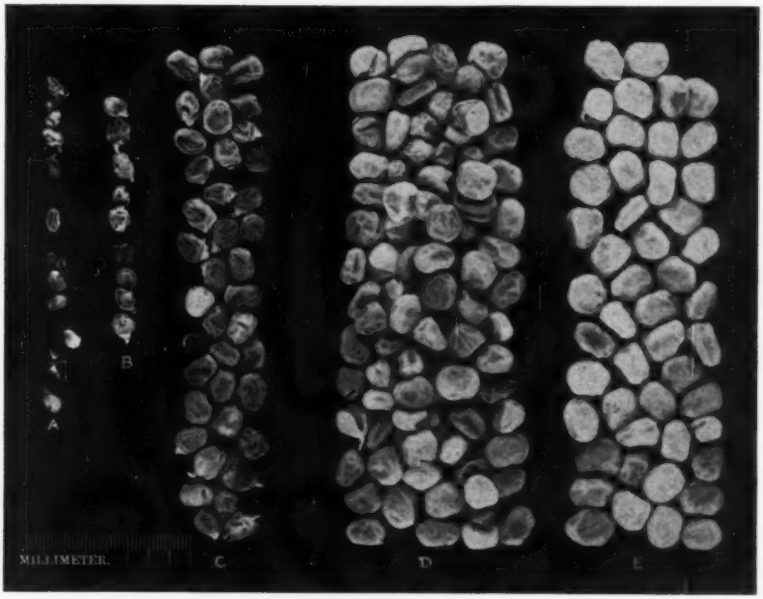

FIGURE 2

PEA SEEDS PRODUCED IN NUTRIENT SOLUTIONS CONTAINING .05 PARTS PER MILLION ZINC (CULTURES 25-30)

The five classes were segregated according to size, color, and plumpness of the seeds (see footnote to Table 2).

ceived .1 ppm. zinc produced a total of 280 seeds, nearly half of which were rated as normal. The next series, although abnormal, produced seeds much like those in the series just below it. The last series, 43-48, which contained 15 plants, produced 271 seeds, more than 2/3 of which fell into the highest class.

While a close point-to-point correlation is not possible, it is perfectly evident that, although the ovules could develop to a certain point with a restricted supply of zinc, they developed into typical seeds only when the supply was above a certain rather well-defined point.

SUMMARY

The growth and reproduction of peas (*Pisum sativum*) is related to the presence of a low, but definite, concentration of zinc in addition to

other nutrient elements. Plants which had additions of less than .01 parts per million of zinc ceased growth and the tops of the majority were dead when they were seven weeks old although the roots were in good condition. Plants which received .05 to .5 ppm. zinc produced approximately the same dry weights of tops and roots when harvested at maturity (14 weeks) but there was an increase in the dry weight of seeds, due to their more complete development, in the higher concentrations of zinc.

Pods and seeds were not produced in cultures which received concentrations of less than .05 parts per million of zinc. The yields suggest that there is a definite threshold value of zinc necessary for the production of seeds.

The stages of development of seeds constitute a convergent line of evidence of the importance of an ample supply of zinc for seed production. Small, shriveled seeds were characteristic of the yields in the lower concentrations, in contrast to the large well-filled seeds in the higher.

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THE REACTION OF MICE WITH SPONTANEOUS TUMORS TO DIPHENYL SULFOXIDE*

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INTRODUCTION

It has been reported that the sulfoxide group may retard the growth of spontaneous tumors in mice through its inhibiting effect on cell proliferation (1-4). Since the tumors continued to grow and the mice to die from the effects thereof it is obvious that sulfoxide in the compounds used, viz., cystine disulfoxide and dl-methionine sulfoxide, has no practical application.

Experience has given the impression that cell increase in number is forwarded by *SH* with greater consistency when this proliferation stimulating group is attached to phenyl—as in para-thiocresol—than it is when attached to an aliphatic radicle as in cysteine. The inference is that phenyl may act as a brake on *SH* oxidation. If this be true it might be that it could also act to retard sulfoxide destruction, and thus allow the group to exert its proliferation inhibiting action more effectively than it does in aliphatic combination.

A fairly decent inhibition of proliferation expression was obtained with aromatic sulfoxides when working with roots of sprouting corn and beans (3). On the basis of these data a trial has been made of the influence of diphenyl sulfoxide on the growth of spontaneous tumors of the mouse.

MATERIALS AND METHODS

The mice used were the inbred descendants of the Bar Harbor "*D*" strain. Thirty-one females with spontaneous tumors served as Controls; 28 as Tests. 0.1 cc. of an acacia-ethanol-solution of diphenyl sulfoxide was administered subcutaneously in the mid-scapular region to the Test mice, daily except Sunday. The mixture had the following composition; 100 mgm. diphenyl sulfoxide, 4 cc. of a 10 per cent solution of gum acacia in distilled water, and 1 cc. of 95 per cent ethanol.

*Aided by a grant from the International Cancer Research Foundation.

This gave a 0.1 molar solution of diphenyl sulfoxide. The same amount of acacia-ethanol stock solution minus the sulfoxide was similarly injected into the Control mice. The sulfoxide was an Eastman product of correct melting point.

Measurements were made of tumor dimensions thrice weekly; and of Body Weight once a week. The usual records of age and survival were kept.

Results

The first two Test mice became paralyzed within one half hour after injection of 0.25 cc. of the diphenyl sulfoxide-acacia-ethanol mixture. Obviously this amount was toxic. The quantity was cut to 0.1 cc. No untoward effects being observed this was the continuing daily dose. One of the paralyzed mice recovered. The other died and is not included in the compilation. Nor are included two mice which died immediately after the 5th and 8th injections respectively with every evidence of respiratory paralysis. Since neither 0.25 cc. nor 0.1 cc. of the acacia-ethanol mixture without sulfoxide produced like evidence of neuro-toxicity in the Control mice, the inference is that the toxic component was diphenyl sulfoxide.

It is usual for spontaneous tumors of the mouse to increase in size

TABLE 1

THE SURVIVAL AND TUMOR VOLUME DATA OF THE CONTROL AND DIPHENYL SULFOXIDE MICE (Averages)

	First tumors			Second tumors			Third tumors		
	Cont.	Test	C-T	Cont.	Test	C-T	Cont.	Test	C-T
Number mice	31	28	3	14	7	7	7	3	4
Percentage	100	100	0	42.5	25.0	20.2	22.6	10.7	11.9
Age; ENTRY	404	362	42	441	438	3	447	450	—3
Tumor; Interval	0	0	0	30	34	—4	20	9	11
Age; DEATH	466	412	54	477	460	17	468	470	—2
Survival	62	50	12	36	22	14	21	20	1
Volume; ENTRY	.268	.376	—.108	.253	.758	—.505	1.044	.249	.795
Volume; MAXIMUM	8.453	7.459	.994	2.475	3.087	—.612	2.387	.879	1.508
Volume; GAIN	8.185	7.083	1.102	2.222	2.329	—.107	1.343	.630	.713
DAYS to Max. Vol.	55	47	8	29	17	12	14	17	—3
Volume; GAIN per DAY	.149	.152	—.003	.074	.135	—.061	.095	.037	.058
Volume; DEATH	6.510	5.624	.886	1.880	2.676	—.796	2.225	.676	1.549
Volume; LOSS	1.943	1.835	.108	.595	.411	.184	.162	.203	—.041
DAYS; Max. Vol. to DEATH	7.4	4.2	3.2	7.0	3.3	3.7	7.0	2.7	4.3
Volume; LOSS per DAY	.262	.437	—.175	.085	.125	—.040	.023	.075	—.052

TABLE 2

AGE, SURVIVAL, AND BODY WEIGHT DATA OF THE CONTROL AND DIPHENYL SULFOXIDE MICE
(Averages)

	Mice with one tumor			Mice with two tumors			Mice with three tumors			All mice combined		
	Cont.	Test	C-T	Cont.	Test	C-T	Cont.	Test	C-T	Cont.	Test	C-T
Number mice	17	21	—4	7	4	3	7	3	4	31	28	3
Percentage	54.8	75.0	—20.2	22.6	14.3	8.3	22.6	10.7	10.7	10.0	10.0	0
Age; ENTRY	396	347	49	421	402	19	405	412	—7	404	362	42
Age; DEATH	457	397	60	486	452	34	468	470	—2	466	412	54
Survival	61	50	11	65	50	15	63	58	5	62	50	12
Weight; ENTRY	26.0	26.5	—0.5	25.0	26.4	—1.4	25.6	27.3	—1.7	25.7	26.6	—0.9
Weight; MAX.	30.6	31.1	—0.5	33.6	31.2	2.4	34.8	33.8	1.0	32.2	31.4	0.8
Weight; GAIN	4.6	4.6	0.0	8.6	4.8	3.8	9.2	6.5	2.7	6.5	4.8	1.7
DAYS; Max. Wt.	46	39	7	59	28	31	55	49	6	51	38	13
Daily Wt. GAIN	.100	.118	—.018	.146	.171	—.025	.167	.133	.034	.127	.126	.001
Weight; DEATH	25.6	26.8	—1.2	28.4	25.1	3.3	29.2	29.6	—0.4	27.0	26.9	0.1
Weight; LOSS	5.0	4.3	0.7	5.2	6.1	—0.9	5.6	4.2	1.4	5.2	4.5	0.7
DAYS; Max. Wt. to DEATH	15	11	4	6	22	—16	8	9	—1	11	12	—1
Daily Wt. LOSS	.333	.391	—.058	.867	.277	.590	.700	.467	.233	.473	.375	.098

to a maximum for a few days before death, and then decrease. The same is true of Body Weight.

Tables 1 and 2 show that the significant facts in this particular experiment are as follows:

1. While maximum tumor volume and tumor gain in volume were generally less in the Tests than in the Controls, growth in size ceased in the Tests before it did in the Controls, and the amount of increase per day while the tumors were growing, was essentially the same in both groups. The same is essentially true for the Body Weight changes.
2. The Test mice did not live as long after tumor induction as did the Controls.
3. The Test mice had fewer secondary tumors than did the Controls; conversely, there were more Control mice with multiple tumors than Test. One mouse in the Control group had four tumors; no mouse in the Test group exhibited such prolificity.
4. The Test mice did not live as long as the Controls after the attainment of maximum tumor volume.
5. The amount of volume loss per day after the attainment of maximum volume was greater in the Tests than in the Controls.
6. Test and Control mice did not differ with respect to survival after the attainment of maximum Body Weight.
7. The daily loss in Body Weight between maximum Body Weight and Body Weight at death tended to be less in the Tests than in the Controls.

Comment

The fact that the span of life after tumor induction was shortened in the mice to which diphenyl sulfoxide was administered despite the fact these mice had fewer and smaller tumors; and the fact that symptoms of paralysis followed by death occurred in some mice shortly after administration of the compound, indicate beyond peradventure that a toxic factor was of considerable moment in the production of the observed results.

Contributory to this interpretation is the fact that the Test mice did not live as long as the Control after the attainment of maximum tumor volume; and the fact that the daily shrinkage in tumor volume during this period was greater in the Tests than in the Controls.

Furthermore the shortening of the span of life of the Test mice cannot be legitimately attributed to tumor growth nor the cachexia

ordinarily incident thereto, since the Test tumors did not get as big as the Control, and the Control mice tended to lose more Body Weight per day than the Test during the interval between the attainment of maximum Body Weight and death.

This outcome was a little unexpected; first because the amount of sulfoxide administered was considerably less than that given in the cystine disulfoxide and the dl-methionine sulfoxide experiments, viz., 2 mgm. as against 8.5 mgm. and 15.5 mgm. of the other two compounds respectively; and second because the toxic reaction was not expressed in any day-by-day retardation of increase in Body Weight or tumor volume.

The fact that Body Weight and tumor volume increase per day during the period of growth was essentially unaffected by diphenyl sulfoxide would seem to indicate there was little if any specific interference with the growth processes concerned in these increments.

On the other hand increase in Body Weight and tumor volume was stopped in the Test mice before it ceased in the Control, and tumor induction in the Test mice was definitely inhibited as shown by the fact that fewer Test mice had multiple tumors.

This lesser incidence of secondary tumors can hardly be attributed to their shorter span of life after first tumor induction, since these mice lived long enough after this event to have gotten second and third tumors had something not stepped in to prevent.

Thus, the Test mice lived on the average for 50 days after first tumor induction, while the Control mice got their second tumors within 30 days after this event, and their third within 43 days. Such being the case it could reasonably be expected that more of the Test mice should have gotten second and third tumors than did.

These two reactions could be interpreted as expressions of inhibition of the proliferation phase of tumor growth by the sulfoxide group (1-4).

If such were true the picture as a whole would be interpreted as a mixed reaction. On the one side there would be the toxic effect presumably attributable to diphenyl; on the other the retardation of cell increase in number unquestionably attributable to sulfoxide (1-4).

But the earlier cessation of tumor volume increase, and the inhibition of multiple tumor production in the Test mice, could as well be taken as expression of toxicity as of specific retardation of proliferative growth.

Such being the case decision is impossible as far as I am concerned, with inclination tending strongly towards toxicity as the major if not sole arbiter of the observed reactions. Contributing to this is the fact that the concentration of sulfoxide was so much smaller than that found expedient to use for retardation of cell increase in number in previous studies of the nature. I am suspicious that insufficient was available to produce its characteristic action. The reader, however, has full privilege to draw his own conclusion.

It is natural of course to wonder why diphenyl sulfoxide should be so toxic. The blame can hardly be put on the sulfoxide moiety as such, since this in other combinations used in larger amounts has not produced like effects. This means that either the diphenyl part of the molecule was the responsible agent, or that the compound was contaminated with some other agent of high toxicity. Whatever the nature of the toxic agent, it was not a growth toxin; but one which seemed to act as a neuro-toxin, inasmuch as its immediate effects were paralysis and convulsion.

Summary and Conclusion

Diphenyl sulfoxide in a daily dosage of 0.01 millimols was administered subcutaneously to 32 female mice with spontaneous tumors. These mice died sooner than their controls; their tumors did not grow as large; they stopped growing sooner; but the amount of increase in tumor volume and body weight per day during the period of growth was no less than that of the controls. Fewer Test mice had multiple tumors. Higher dosage killed the mice with symptoms of neurotoxicity. The conclusion is that diphenyl sulfoxide in the amount administered, viz. 0.1 cc. of a 0.1 molar solution, is slowly and lethally toxic to mice bearing spontaneous tumors.

These experiments were carried on under the supervision of Dr. Gerrit Toennies whose generous assistance is gratefully acknowledged.

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THE BIOSYNTHESIS OF THIAMINE BY NORMALLY ATHIAMINOGENIC MICROORGANISMS[1]

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A complete investigation of the growth requirements of any microörganism necessitates the accomplishment of a pure culture (free from other living organisms) and the formulation of a chemically known basic medium. Our knowledge of the growth requirements of the protozoa has been limited largely to the flagellates because of the second mentioned limiting condition. So far no ciliate has been cultured in a medium of known chemical composition. They seem to require whole proteins or protein degradation products as a source of nitrogen (Lwoff, 1932; Dewey, 1942). It is possible, however, to determine some of the supplementary requirements of those ciliates which will grow in pure culture provided the basic protein or peptone medium can be rendered free of the substance under investigation (Lwoff and Lwoff, 1937). Due to the lability of thiamine (vitamin B_1) investigations of this requirement have been undertaken in the past, the investigators heat-treating their basic protein or peptone media in the presence of alkali. It has been reported that *Tetrahymena geleii* requires an exogenous source of thiamine (Lwoff and Lwoff, 1937; Elliott, 1939; Hall, 1939; Dewey and Kidder, 1941; Baker and Johnson, 1941). In addition to this species we (Dewey and Kidder, 1941) reported a like requirement for *Tetrahymena vorax* and *Glaucoma scintillans*. The present paper deals with an extension of the previous preliminary report and shows that both *Tetrahymena geleii* and *T. vorax* are able to construct the pyrimidine and thiazole intermediates and, under certain conditions, the thiamine molecule. *Glaucoma scintillans* lacks these synthetic powers.

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MATERIAL AND METHODS

1. *Microörganisms*

Tetrahymena geleii (Strain *W*) is the strain which has been used in this laboratory in previous studies (Kidder, 1941; Dewey, 1941, 1942). This species has been shown to require proteins or peptones as a source of nitrogen and has not been grown in mixtures of pure amino acids (Lwoff, 1924, 1932; Dewey, 1941, 1942). It has been shown to require, in addition to an exogenous source of thiamine, two substances, referred to as Factors I and II (Dewey, 1942). The basic medium was 1 per cent vitamin-free casein (Harris, highest chemical purity) supplemented with heat- and alkali-treated concentrates of Factors I and II or water extracts of timothy or alfalfa. The concentrates and extracts were heated to 123° C. for 1-7 hours at pH 10-11.5. In some cases heat- and alkali-treated proteose-peptone (Difco) was substituted for the casein.

Tetrahymena vorax is the strain used in the studies of Kidder, Lilly, and Claff (1940) and Kidder (1941). In its saprozoic state its requirements are like those of *T. geleii* (Dewey, 1942) and the basic media are the same.

Glaucoma scintillans (Strain *A*) is the strain used in the growth studies of Kidder (1941). At that time it was reported that this species required specific substances from yeast and was only able to grow in a particulate medium. Since the publication of those results a variant strain has been obtained from the original which grows well in proteose-peptone or in casein supplemented with Factors I and II. The problem of the origin of this variant strain will be discussed in a later paper.

Phycomyces blakesleeanus was obtained from Dr. W. J. Robbins. This fungus has been shown to be able to grow in a synthetic medium

if both the pyrimidine and thiazole components of thiamine are present (Robbins and Kavanagh, 1937; Schopfer, 1937). The basic medium used in the present study was "Medium *C*" of Robbins (1938).

Phytophthora cinnamomi was also obtained from Dr. Robbins. This species has been reported by Robbins (1938) to be unable to synthesize the thiamine molecule from its components but must be supplied with intact vitamin B_1 for optimum growth. The basic medium used was also "Medium *C*."

2. *Substances Used in the Preparation of Media*

The following substances and pure chemicals were used in this study; proteose-peptone (Difco); casein (Harris, highest chemical purity); alfalfa meal (Denver Alfalfa Milling and Products Co.); liver extract (Difco); yeast extracts (Difco and Harris Yeast Vitamin Concentrate); thiamine hydrochloride (Hoffman-LaRoche); pyrimidine (2-methyl-5-ethoxymethyl-6-amino-pyrimidine), thiazole (4-methyl-5-Beta-hydroxyethyl thiazole), pyridoxin, pantothenic acid and riboflavin (Merck and Co.); nicotinic acid, pimelic acid, i-inositol, uracil and p-aminobenzoic acid (Eastman Kodak Co.); biotin methyl ester (S. M. A. Corp.); inorganic salts (Baker and Adamson).

3. *Methods*

Pyrex glassware was used exclusively. All glassware was cleaned by soaking for one hour in a saturated solution of trisodium phosphate or a similar substance going under the name of "Keego." After a thorough rinsing in tap water it was given a number of rinses in double (Pyrex) distilled water.

Water used in making media was only that which had been distilled twice over permanganate in an all Pyrex still.

For the qualitative results reported here we have used the tube method of serial transplant. All data are based on the results of the growth in the third transplant. The importance of this method is stressed elsewhere (Lwoff, 1932; Dewey, 1942). The results represent growth in the experimental tubes as compared to control tubes in which optimum growth occurred. The number of plus signs represent a graded evaluation from optimum (++++) with plus-minus (±) being barely perceptible (under the conditions and duration of these experiments) but transplantable growth, while zero (0) means no growth in the third transplant. Inasmuch as we were interested

in virtually all-or-none reactions the times allowed between transplants were as follows: *Tetrahymena geleii*—48 hours; *T. vorax*—72 hours; *Glaucoma scintillans*—96 hours; *Phycomyces blakesleeanus* and *Phytophthora cinnamomi*—10 days. Transplants were made, in the case of the protozoa, with a bacteriological loop while in the case of the fungi a straight bacteriological needle was used to transfer small bits of mycelium.

Incubation was at room temperature and all organisms were in pure culture at all times.

Experimental

It has been shown (Dewey, 1941; 1942) that there are at least two supplementary growth substances required by *Tetrahymena geleii* and *T. vorax* which are present in normal peptone solutions, egg yolk, milk, yeast, and various higher plants. Using a vitamin-free casein it was found that very little growth occurred upon the addition of any of the known vitamins but when a water extract of such materials as timothy hay or alfalfa was added an immediate response was shown and optimum growth occurred. Moreover Dewey (1941; 1942) was able to fractionate these extracts by various methods and she designated the two active protein-free fractions obtained as Factors I and II. It was found that both factors were present in peptones but Factor I was destroyed by prolonged heat while neither factor obtained from plant sources was in the least heat labile. The variant strain of *Glaucoma scintillans* also requires Factors I and II.

Taking advantage of the extreme chemical stability of Factors I and II from plant sources we have been able to show that there occurs a third factor (which we will call Factor *S*) in plant material which, when present in the medium along with Factors I and II makes the inclusion of thiamine unnecessary for the optimum growth of either species of *Tetrahymena*, but does not function in that manner for *Glaucoma scintillans*.

The first exploratory experiments were conducted with a hot water extract of alfalfa meal (5 per cent by weight). This extract was made very alkaline (pH 10-11.5 as determined by the hydrogen electrode) with NaOH and heated at 123° C. for one hour or more. This treatment was used to insure the destruction of all thiamine and we know that most, if not all, of the thiazole portion of the molecule must also be destroyed by such treatment. After treatment the pH was

TABLE 1

THE EFFECT OF HEAT AND ALKALI TREATED ALFALFA EXTRACT ON THE GROWTH OF *Tetrahymena* AND *Glaucoma*

	1	2	3	4	5	6	7	8
	HPP	*C*	*HPP* B_1	*C* B_1	*HPP* *A*	*C* *A*	*HPP* *A* B_1	*C* *A* B_1
T. geleii	0	0	+	+	++++	++++	++++	++++
T. vorax	0	0	0	0	++++	++++	++++	++++
G. scintillans	0	0	0	0	0	0	++++	++++

HPP = Proteose-peptone autoclaved 1 hr. at pH 10-11.5; *C* = 1% vitamin-free casein; B_1 = 0.05 mg/% thiamine hydrochloride; *A* = water extract of alfalfa meal (0.5%) autoclaved 7 hours at pH 10-11.5.

adjusted to 6.8-7.0 with HCl. Using this heat-treated extract (designated *A*) as a supplement to either 1 per cent proteose-peptone (heat and alkali treated as above) or 1 per cent casein it was discovered that both species of *Tetrahymena* grew equally well either with or without the addition of thiamine. *Glaucoma scintillans,* however, grew only when thiamine was added (Table 1).

There appeared to be a number of alternative explanations for these results: (*a*) the alfalfa contained a stable substance which could act as a substitute for thiamine in the case of *Tetrahymena* but not *Glaucoma;* (*b*) thiamine is not a requirement for *Tetrahymena* but is for *Glaucoma.* All that is needed, therefore, would be the stable Factors I and II. This would be in disagreement with the accepted data; (*c*) The alfalfa extract contains a stable substance which catalyzes the synthesis of thiamine in the case of *Tetrahymena* but is ineffective for *Glaucoma.* If the last alternative is true then there is still the question of the synthesis factor (Factor *S*) being identical with either Factor I or Factor II or some other known vitamin (however, none of the known growth substances listed under materials was effective singly or in combinations).

It was shown by the following experiments that *Tetrahymena* actually synthesizes thiamine. Casein was supplemented with *A* ("dethiaminized" alfalfa extract) and *Tetrahymena geleii* was grown in this medium through the usual three transplants. Medium which had supported the growth of a population was treated in various ways: (*a*) the population of cells was removed by filtration; (*b*) the cells were removed by centrifugation; (*c*) the cells were allowed to remain but were killed by heating in the autoclave; (*d*) the cells were killed by immersing the tubes in boiling water for one minute. *Glaucoma* were transplanted into these conditioned media which previously would not support its growth. The results are shown in Table 2.

It seems conclusive from the above results that Alternative 1 is not

TABLE 2

GROWTH OF *Glaucoma scintillans* IN *Tetrahymena geleii*-CONDITIONED MEDIUM

Unconditioned		Conditioned ($C + A$)			
C A	C A B_1	Filtered	Centrifuged	Autoclaved	Heated 1 min.
0	++++	0	0	++++	++++

C = 1% vitamin-free casein; A = water extract of alfalfa meal (0.5%) autoclaved 1 hour at pH 11.5; B_1 = 0.05 mg/% thiamine hydrochloride.

correct and that thiamine has been synthesized (Alternative 3). It is also demonstrated that thiamine is not released into the medium. It still might be true that *Tetrahymena* synthesizes thiamine but does not utilize it (Alternative 2). This is unthinkable at the present time in view of the work of Lwoff and Lwoff (1937). However, further tests were prepared to demonstrate that exogenous thiamine is required in the absence of Factor *S* and in the presence of Factor I and Factor II. It had been shown previously (Dewey, 1942) that heat and alkali treated proteose-peptone, even with added thiamine, would not support the growth of *Tetrahymena* unless Factor I (as a concentrate, certainly containing thiamine) was added. This leads to the conclusion that ordinary peptone contains Factor II in a heat stable form and Factor I (or something which will take its place) in a labile form. When Factor I and Factor II are separated by lead acetate precipitation all of the Factor *S* goes with Factor II (fails to be precipitated). Tests had shown that Factor *S* was not found in materials from animal sources. Therefore combining heated peptone (containing Factor II) with the factor precipitated by the salts of the heavy metals (Factor I), in which the thiamine had been destroyed by heat and alkali treatment, should give information regarding the necessity of the thiamine molecule for *Tetrahymena*. Table 3 sums up experiments of this type.

TABLE 3

GROWTH OF *Tetrahymena geleii* WITHOUT FACTOR *S*

1	2	3	4
C *HPP*	*C* *PbP*	*C* *HPP* *PbP*	*C* *HPP* *PbP* B_1
0	0	0	++++

C = 1% vitamin-free casein; *HPP* = heat-treated proteose-peptone (contains Factor II); *PbP* = fraction precipitated by lead acetate from alfalfa extract (contains Factor I); *B* = 0.05 mg/% thiamine hydrochloride.

This indicates that when the synthesis of thiamine is blocked no growth of *Tetrahymena* occurs unless an outside source of the vitamin is supplied. It also suggests that Factor *S* is distinct from Factors I and II, at least if it is true that the Factor I of animal material is identical with the Factor I of plant material.

At this point it seems that the following statement is correct: *Tetrahymena geleii* and *T. vorax* need, as supplementary growth factors,

Factor I and Factor II and thiamine. Thiamine can be synthesized in the presence of Factors I and II if a third factor (Factor *S*) is present. *Glaucoma*, on the other hand, lacks the mechanism for thiamine synthesis, even in the presence of all three factors, hence must always be supplied with exogenous thiamine.

Having the above evidence for the existence of a "synthesis factor" for *Tetrahymena* and also as an added check on the effectiveness of our heat treatment in destroying thiamine, we decided to test its effectiveness on an entirely different type of organism. According to the beautiful work of Robbins (1938) the mold *Phytophthora cinnamomi* fails to grow in the absence of an outside source of thiamine. This was corroborated, at least regarding the production of optimum growth with aerial hyphae, in our experiments using Robbin's medium *C*. The addition of either pyrimidine or thiazole or the two together to the basic medium made only slight difference, but the addition of the whole thiamine molecule produced optimum growth. The addition of alfalfa extract (heat and alkali treated) to these media gave results as are shown in Table 4.

TABLE 4

GROWTH OF *Phytophthora cinnamomi*

The results represent third transplants and are taken from a number of experiments.

1	2	3	4	5	6	7	8	9
C	*C* + *T*	*C* + *P*	*C* + *T* + *P*	*C* + *A*	*C* + *T* + *A*	*C* + *P* + *A*	*C* + *T* + *P* + *A*	*C* + B_1
0	±	±	+	±	+++	±	++++	++++

C = Medium *C* of Robbins (1938); *T* = 0.025 mg/% thiazole; *P* = 0.025 mg/% pyrimidine; *A* = 0.5% heat and alkali treated water extract of alfalfa meal; B_1 = 0.05 mg/% thiamine hydrochloride.

This type of experiment indicates quite clearly that Factor *S* functions equally well for *Phytophthora* and for *Tetrahymena*, for if thiamine had still been present after our treatment then growth should have been good in Medium 5 (Table 4). It also indicated that our treatment failed to destroy the pyrimidine component but was effective in thiazole destruction (compare Column 6 with Columns 7 and 8 in Table 4).

It was decided that information of value might be obtained regarding the level at which synthesis takes place under the influence of Factor *S* if treatment was given to destroy both components of the thiamine mol-

ecule—in other words to render the alfalfa extracts pyrimidine—and thiazole-free. Accordingly a heat and alkali treated extract was placed in a water tight jacket surrounding the tube of a Sterilamp and the material (in contact with the tube) irradiated for varying lengths of time. The ultra violet treatment was suggested by the work of Uber and Verbrugge (1940a; 1940b). Material was irradiated one hour, 4 hours, 14 hours, and 66 hours and the tests were run using *Phycomyces blakesleeanus* for the detection of pyrimidine and thiazole. *Phytophthora cinnamomi* was also used in these experiments as a check on the results of the preceding set. Table 5 indicates the results.

The experiments with the irradiated alfalfa indicate conclusively that Factor *S* is effective at the level of synthesis where the pyrimidine and thiazole components are combined to form the thiamine molecule. *Phytophthora cinnamomi* did not grow appreciably in the presence of Factor *S* alone nor in the presence of Factor *S* and either of the intermediate components singly, but gave optimum growth when both pyrimidine and thiazole were present with Factor *S*. The tests with *Phycomyces blakesleeanus* proved that both components were destroyed, under our conditions, after four hours of irradiation.

Using the ultraviolet-treated extract as a source of Factor *S* it was shown that the addition of the pyrimidine and thiazole components either singly or together made no difference in the growth of either species of *Tetrahymena*. This would indicate that these ciliates normally synthesize the components but, without Factor *S*, are unable to construct the thiamine molecule. These experiments also demonstrated two other points: (*a*) the progressive destruction of Factor I by the radiation (noted by Dewey, 1942) and, (*b*) the higher requirement for Factor I exhibited by *Tetrahymena vorax* as compared to *T. geleii*. Table 6 is a summary of the above experiments.

We have tested a number of raw materials for the presence of Factor *S*, using the general methods outlined above. So far it has been impossible to decide whether or not some of these materials possess the factor or whether the heat and alkali treatment has caused the production of toxic substances which influence adversely the results of our tests. That toxic substances are produced is evident in a number of cases and when these occur they may mask the presence of Factor *S*. From our survey we can say, however, that Factor *S* is present in highest concentrations in the leaves of higher plants, and not detectable by our methods in material of animal origin. The materials which we have

TABLE 5

TEST FOR THIAZOLE AND PYRIMIDINE IN ULTRAVIOLET IRRADIATED ALFALFA EXTRACT WHICH HAD PREVIOUSLY BEEN HEATED AND ALKALI TREATED

Results of third transplants from four separate experiments.

	1	2	3	4	5	6	7	8	9
	C	*C* *T*	*C* *P*	*C* *T* + *P*	*C* B_1	*C* *AU*	*C* *T* + *AU*	*C* *P* + *AU*	*C* *T* + *P* *AU*
Phycomyces	0	±	±	++++	++++	0	±	±	++++
Phytophthora	0	±	±	±	++++	0	±	±	++++

C = Medium C of Robbins (1938); *T* = 0.025 mg/% thiazole; *P* = 0.025 mg/% pyrimidine; B_1 = 0.05 mg/% thiamine hydrochloride; *AU* = 0.5% heat and alkali treated water extract of alfalfa meal irradiated with ultraviolet for 4 hours in two experiments and 66 hours in two experiments, no differences between two sets.

TABLE 6

	Tetrahymena geleii	Tetrahymena vorax
Control: 1% Casein as Base	0	0
Base + Thiamine	0	0
Base + *A*	++++	++++
Base + Thiazole and Pyrimidine	0	0
Base + *AU* 1 hr.	+++	++
Base + *AU* 1 hr. Thiazole + Pyrimidine	+++	++
Base + *AU* 1 hr. Thiamine	+++	++
Base + *AU* 4 hr.	+++	+
Base + *AU* 4 hr. Thiazole + Pyrimidine	+++	+
Base + *AU* 4 hr. Thiamine	+++	+
Base + *AU* 14 hr.	++	0
Base + *AU* 14 hr. Thiazole + Pyrimidine	++	0
Base + *AU* 14 hr. Thiamine	++	0
Base + *AU* 66 hr.	+	0
Base + *AU* 66 hr. Thiazole + Pyrimidine	+	0
Base + *AU* 66 hr. Thiamine	+	0
Base + *AU* 66 hr. *A* (as a control on toxicity)	++++	++++

A = heat and alkali treated water extract of alfalfa meal; *AU* = "*A*" radiated with ultraviolet the number of hours indicated; Thiamine = 0.05 mg/%; Thiazole and Pyrimidine = 0.025 mg/%.

tested may be placed in four categories: (*a*) those having large amounts of Factor *S* (alfalfa leaf, timothy leaf, grass, and lettuce); (*b*) those having appreciable amounts of Factor *S* (alfalfa stem and timothy head); (*c*) those having barely detectable amounts of Factor *S* (timothy stem, potato peel, rice bran, and yeast. It is probable that the yeast estimates are not correct but that the production of toxic

substances was high); (*d*) those having no detectable amounts of Factor *S* (potato, liver, milk serum, whole milk, peptone, meat extract).

Factor *S* may be tentatively characterized by its solubility in water and alcohol up to 75 per cent, insolubility in acetone and ether, stability to prolonged heat either in the presence of alkalis or acids, and its stability to ultraviolet radiation. It can be dialysed through cellophane and is not precipitated by the salts of heavy metals. It appears to be adsorbed on Norit and, to some extent, on Fuller's earth but is not adsorbed on Cellite or Supercell. Purification of this factor is being attempted and until this is accomplished the results can be expressed only in qualitative terms.

Discussion

The distribution of Factor *S* seems to be correlated with the power of the source organism to perform thiamine synthesis. It is well known that thiamine is manufactured in the leafy parts of most higher plants and it is important to note that Factor *S* is found in abundance only in the leaves. Higher animals lack thiamine synthetic powers and Factor *S* was not found in any of the materials of animal origin tested.

We do not know what the mechanisms are which are utilized by thiamine-synthesizing organisms but it may well be that the reactions involved depend first on the ability to manufacture the intermediates, and second on a catalyst for their combination into the thiamine molecule. We are inclined, at the present time, to look upon Factor *S* as the latter type of catalyst.

Of course *Glaucoma scintillans* must be considered more complex, since this organism lacks the power to combine the intermediates into the thiamine molecule even in the presence of the catalyst. Following Lwoff's (1932) reasoning, therefore, *Glaucoma* has lost synthetic powers still possessed, to a degree, by *Tetrahymena*, and represents a further step in physiological evolution.

It is quite evident that the powers of synthesis for either or both of the thiamine intermediates, pyrimidine and thiazole, need not be intimately associated with the ability of the organism to construct the thiamine molecule. *Phytophthora cinnamomi* is unable to synthesize appreciable amounts of either component but is able to construct the thiamine molecule in the presence of the catalyst, Factor *S*. *Phycomyces blakesleeanus* has similar limitations for the intermediates, but prob-

ably constructs its own catalyst. Although we have not tested this point, it would not be surprising to find that extracts of *Phycomyces* contain Factor *S* while those of *Phytophthora* do not.

From the experiments cited above we realize that, although *Tetrahymena* and *Phytophthora* are alike in their requirements for either exogenous thiamine or Factor *S*, they differ in their intermediate synthesis. *Tetrahymena* apparently has retained more synthetic ability in this respect than has *Phytophthora*. The failure of Factor *S* to influence *Glaucoma scintillans* makes it impossible to judge whether or not the powers to synthesize pyrimidine and thiazole have been lost by this organism.

It will be interesting and important to see whether there are other normally "athiaminogenic" animals which will be affected by Factor *S*. The approach to this much broader field should await the purification of the specific substance.

Summary

1. Three ciliated protozoa and two fungi were used in this investigation.

2. Two of the ciliates (*Tetrahymena geleii* and *T. vorax*) were found to be able to synthesize thiamine if a substance from leaves, Factor *S*, was added to a medium free of thiamine or its intermediates.

3. The third ciliate (*Glaucoma scintillans*) was in all cases, found to require exogenous thiamine for growth.

4. The mold *Phytophthora cinnamomi* was able to synthesize thiamine in the presence of Factor *S*, but only when the thiamine intermediates were supplied.

5. By the use of the growth characteristics of *Phycomyces blakesleeanus* it was shown that both the pyrimidine and the thiazole components of the thiamine molecule could be destroyed by ultra violet radiation in four hours (under the conditions of our experiments).

6. Factor *S* has been found only in material from plant sources. It is more concentrated in the leaves of the higher plants than elsewhere.

7. Factor *S* is a highly stable substance which is postulated as being capable of catalyzing the reaction for the formation of the thiamine molecule from its intermediates. It does not appear to be connected with the synthesis of the thiamine intermediates.

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DWARFISM: DIAGNOSTIC CRITERIA AND ENDOCRINE THERAPY

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There have been many attempts to influence human growth by utilizing various so-called pituitary growth extracts. These have resulted in various degrees of response. Most of these growth preparations have been relatively impotent and it was partly on this assumption that the cases herein reported were treated with large and frequent dosages, in order to approach optimal effect.

Attempts to augment the action of these extracts have been made by many investigators. Collip (4) in 1934 was able to effect a more rapid weight gain in hypophysectomized rats utilizing one of his purified anterior pituitary preparations. Smith (26) showed that when thyroid was administered to hypophysectomized animals there was no stimulation of growth; however, when thyroid and anterior pituitary growth extracts were given concurrently, the growth response exceeded that obtained when the pituitary extract was given alone. This experiment was subsequently elaborated and confirmed by Evans, Simpson, and Pencharz (12).

Dorff (6, 7) claimed a marked growth stimulation with chorionic gonadotropic extracts in patients who were moderately tall and those who were stunted and attributed the results to gonadal stimulation which in turn stimulated the pituitary leading to an increased output of growth factor. He found that in boys in whom treatment was instituted before the age of 13 years the growth response was particularly good, but as a rule, there was no significant acceleration in the rate of growth in those in whom therapy was started between the ages of 13 and 15 years. It was in the three cases in this age-group that the greatest growth increases were observed. Furthermore, chorionic gonadotropic factor may stimulate growth by precipitating the "pubertal spurt" normally seen later. The ultimate total growth with this type of treatment may not be affected. The growth-promoting factor

of the pituitary has been found to produce growth in static individuals, which otherwise might not have occurred.

The authors feel that the statement that no potent anterior pituitary products are available for the stimulation of growth is not warranted, as the majority of cases of dwarfism in which growth responses has been elicited have been treated with this type of preparation (13, 8, 9, 25, 27, 18, 19). On the other hand, Yarvis (30) reported that the rate of growth was not affected in a boy treated with chorionic gonadotropic factor, in whom a marked stimulation of growth had been previously accomplished with pituitary growth factor and thyroid therapy.

We have been unable to find any clinical reports on the stimulation of growth in the achondroplasias with the use of pituitary growth extracts. However, Evans, Simpson, Meyer, and Reichert (11) have shown that in the dachshund, which was considered as a breed of dog having normal skull and vertebral column but with a marked achondroplasia in the legs, the prolonged administration of growth extract produced a marked gigantism and an increase in the length of the animals due to an increase in the size of the skull and vertebrae.

The present investigation has been extended to include human cases of achondroplasia, in order to ascertain whether any acceleration in the rate of growth would result from pituitary growth extract therapy.

The decision to treat a given case was not made without difficulty. It is the opinion of the authors that the pituitary not only plays a prominent rôle in growth, but that every type of dwarfism probably constitutes some sort of pituitary deficiency. It is imperative however, that one should be aware of such contributing factors as hypothyroidism, infection, parasitic infestation, osteochondrodystrophy, coeliac disease, etc., as these influence the growth action of the anterior pituitary.

Many types of pituitary preparations containing the alleged growth-promoting principle have been clinically and experimentally utilized. The nature of this so-called growth hormone is not well understood. However, it has been adequately demonstrated both clinically and experimentally that these extracts contain a growth-promoting activity. We do not feel qualified to answer the question as to whether there is a specific growth hormone or whether this activity is associated with one of the other factors.

The preparation utilized in this investigation undoubtedly contained many of the other tropic factors as well as inert protein. It is highly probable that more purified products would produce a greater response

as many of these contaminating factors are physiologically antagonistic. Despite this, the preparations utilized were definitely potent, as they had been demonstrated to stimulate growth in hypophysectomized animals.

In the cases of Lorain-Levi hypopituitary dwarfism, which were observed over a period of several years, the following criteria were established:

(*a*) Normally proportioned bodily configuration with a retardation in height.

(*b*) High-arched palate.

(*c*) Small lower jaw with crowding of teeth.

(*d*) Tapering fingers and freely movable joints.

(*e*) Normal or elevated *IQ* with a child-like demeanor.

(*f*) Normal sella (two cases with small sellas due to bridging of the clinoid processes were seen).

(*g*) Normal blood constituents and normal B.M.R.

(*h*) Bones osteologically normal but with delay in epiphyseal closure.

Infantilism may or may not be present. In our experience, epiphyseal union was usually not delayed in cases where genital development was proceeding normally. This found some substantiation in the work of Gudernatsch (16) and Hammett (17), who demonstrated that tissue differentiation and tissue proliferation were antagonistic.

Cases of hypopituitary obesity have been encountered in which there was also a growth deficiency. These cases presented the typical configuration of the Fröhlich syndrome. These were selected for growth therapy.

Achondroplasia can be diagnosed early in life. There is a congenital abnormality of cartilaginous bone formation resulting in a deficiency in growth of the long bones, with a marked disproportion between the trunk and the limbs, giving the child its characteristic appearance.

In determining growth standards, the height charts published by Burgess (3) were utilized. According to Burgess, the greater the rate of growth, the earlier the plateau is reached; and in those cases in which growth progresses slowly, it usually continued beyond the average pattern. An individual who has grown along a certain percentage curve will tend to adhere to that curve.

The plan adopted in studying our cases was similar to that advocated by Watson and Moehlig (28). Included in all cases was a careful

history, physical examination and anthropometric measurements which were diagrammatically depicted on a staturogram (24) (Figure 1).

AGE (YEARS)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
HEIGHT (INCHES)	♀	29.0	32.4	34.7	37.0	41.2	44.4	46.8	48.9	50.9	53.0	55.2	57.0	59.7	61.4	62.5	63.2	63.7	64.0	64.0	64.0
	♂	29.5	32.9	35.3	38.1	42.7	45.0	47.4	49.2	51.2	53.1	55.2	57.1	58.9	60.7	62.4	64.0	65.4	66.9	67.5	68.0
WEIGHT (POUNDS)	♀	30.1	36.7	39.8	44.0	47.7	52.0	57.4	63.6	71.4	80.6	90.6	101.4	107.7	110.9	114.2	117.6	117.8	117.8	117.6	
	♂	31.4	37.2	41.0	45.6	49.3	52.4	58.1	64.6	70.8	77.8	85.3	95.0	98.0	101.4	106.0	111.7	114.6	116.4	118.6	118.0
SPAN (INCHES)	♀	27.7	32.1	35.1	38.1	40.7	43.5	46.0	48.1	50.4	52.6	52.8	55.5	59.1	61.3	63.0	63.6	64.2	64.8	64.8	64.8
	♂	28.3	32.7	36.8	38.8	41.4	44.0	46.1	48.6	51.0	53.4	55.6	57.9	60.2	61.9	64.1	65.8	67.5	68.0	69.8	70.4
HEAD (INCHES)	♀	18.2	19.0	19.4	19.7	20.0	20.1	20.3	20.4	20.6	20.7	20.9	21.0	21.2	21.4	21.6	21.7	21.8	21.8	21.9	21.9
	♂	18.6	19.4	19.8	20.1	20.4	20.5	20.6	20.7	20.9	21.0	21.1	21.2	21.4	21.6	21.8	22.0	22.1	22.4	22.5	22.5
CHEST (INCHES)	♀	18.1	19.2	20.0	20.7	21.4	22.0	22.7	23.4	24.2	25.0	26.1	27.1	28.1	28.9	29.6	30.1	30.5	30.8	30.9	31.0
	♂	18.5	19.7	20.6	21.4	22.1	22.7	23.5	24.0	24.6	25.3	26.1	27.0	28.0	28.9	29.8	30.3	31.7	32.9	33.7	34.4	34.7
WAIST (INCHES)	♀	17.0	18.0	18.1	18.5	19.0	20.2	20.3	20.8	21.2	21.8	22.4	23.2	23.8	24.5	24.8	25.1	25.3	25.5	25.6	25.7
	♂	17.9	18.9	19.5	20.0	20.4	20.9	21.5	21.8	22.3	22.8	23.3	23.9	24.6	25.4	26.6	27.2	27.8	28.2	28.9	28.7
UPPER (INCHES)	♀	17.8	19.6	20.8	22.0	22.8	23.6	24.4	25.1	25.8	26.7	27.8	28.7	29.6	30.4	31.2	31.8	31.8	32.1	32.1	32.1
	♂	17.9	20.1	21.5	22.4	23.3	24.0	24.6	25.1	25.9	26.7	27.4	28.3	29.1	29.8	30.8	31.5	32.2	32.6	33.4	33.7
LOWER (INCHES)	♀	11.8	13.6	15.7	17.3	19.2	20.9	21.8	23.0	24.1	25.2	25.7	25.8	25.5	25.3	25.3	25.8	26.7	27.6	27.9	27.9
	♂	11.6	13.6	15.7	17.6	19.2	21.0	22.4	23.8	25.1	26.3	27.6	28.7	29.8	30.7	31.7	32.5	33.2	33.7	34.1	34.3

FIGURE 1

NORMAL VALUES IN INCHES (ENGELBACH) UTILIZED IN CONSTRUCTING STATUROGRAM

The body measurements were compared with those established by Engelbach (10). Appropriate laboratory studies were carried out. The bones were *x*-rayed periodically for the determination of bone age and epiphyseal union. Roentgenograms of the sella turcica were also obtained. Photographs were taken before treatment was instituted and at various intervals thereafter.

TREATMENT AND RESULTS

If the height of the patient when first seen was below the 1 per cent curve of Burgess's growth chart, treatment was instituted regardless of age. If the height was between the 5 per cent and 30 per cent curve and the child was under six years of age, a preliminary observation period was carried out for 1-2 years during which time the child was placed on a highly nutritious diet with measurements plotted at six months intervals. If at the end of this period the growth increment was 50 per cent or more below normal expectancy, appropriate therapy was carried out.

Treatment was designed to approximate the conditions which resulted in optimal growth response in animals. In most instances it was given daily or on alternate days in doses varying from 1 to 10 cc., with an average dose of 3 cc. The growth extract (G. F.)[1] used was an

[1]The growth extracts employed in this study were generously supplied by Ayerst, McKenna, and Harrison, Ltd., Montreal, Canada (Growth Complex, shown in the text as G. F.)

alkaline extract of beef hypophysis. The material was standardized to contain 100 Collip Rat Units of growth-promoting activity per cc. In addition to this material, a special preparation of growth extract (S. G. F.)[2] was utilized in similar dosages. The latter preparation was prepared by Dr. Cook and assayed well over 100 Rat Units per cc. The so-called thyrotropic factor[3] was added later to determine whether an augmentative effect could be demonstrated. In most instances thyroid extract was utilized when thyrotropic factor was not available. In addition, supportive therapy consisting of 15-22 gr. of dicalcium phosphate with viosterol, 5-10 mg. of thiamin chloride, ultraviolet irradiation, essential growth amino acids[4] and a high-caloric, high-protein diet was utilized.

1. *Case 1*

A. C. was a 14½ year old female weighing 60 pounds, and 53 inches in height, on the initial visit. The early childhood history was essentially negative. Her appetite was adequate but it was difficult for her to gain weight. Menarche had not yet occurred. Her intelligence quotient was above normal. In the 12 months prior to treatment she had grown only 1¾ inches.

Examination revealed a slender, normally proportioned, white female of short stature, corresponding to that of a child of 11 years of age (Figure 2). The lower teeth were crowded and indicative of an underdeveloped jaw. Axillary and pubic hair and mammary gland development were absent. Other physical findings were essentially negative. The lower body measurement exceeded the upper, and the span exceeded the height (Figure 3). The epiphyses were not fused and the bone age was equivalent to that of a child of 11 years. *X*-ray of the sella was normal. The B. M. R. was plus 13. A diagnosis of pituitary infantilism was made.

Treatment was instituted with commercial growth factor (G. F.) (Figure 4). After nine months of therapy the epiphyses were *x*-rayed again and found to be open. After a year of therapy menarche took place and menses occurred at 28-30 day intervals thereafter, with subsequent development of secondary sex characteristics. There was a growth of four inches in 18 months of treatment as compared to an

[2]Generously furnished and prepared by Dr. Cook of Ayerst, McKenna and Harrison, Ltd., Montreal, Canada. (Special Growth Complex, as shown in the text as S. G. F.)

[3]The Thyrotropic Factor was also supplied by Ayerst, McKenna, and Harrison, Ltd., Montreal, Canada.

[4]Supplied by the Arlington Chemical Company, Yonkers, New York, as Aminoids.

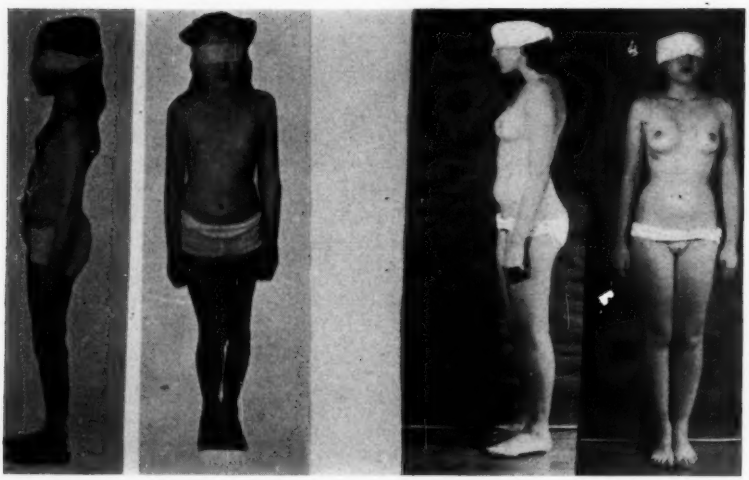

FIGURE 2
CASE 1 BEFORE, AND 1½ YEARS AFTER THERAPY WITH GROWTH EXTRACT

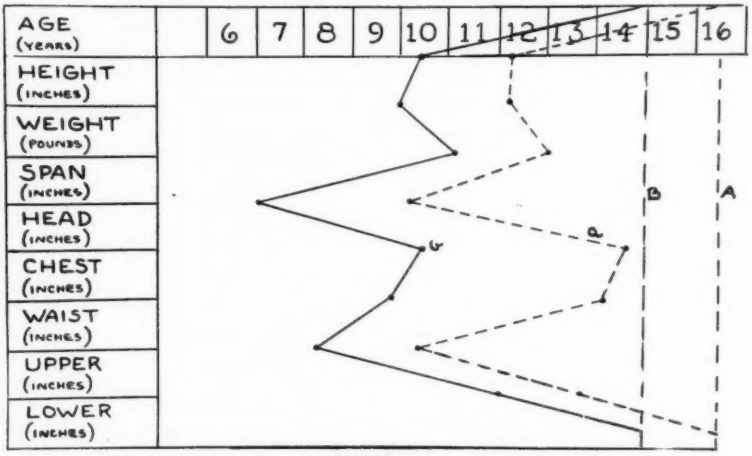

FIGURE 3
CASE 1: STATUROGRAM INDICATING NORMAL DEVELOPMENT (*A* TO *B*) AS COMPARED TO ACTUAL DEVELOPMENT (*a* TO *b*) UNDER GROWTH THERAPY

		CASE 1.	CASE 2.	CASE 3.	CASE 4.	CASE 5.
3 Mos.	G.F. DOSE	5750 R.U. 4cc. 3 x week	6950 R.U. 3 x week	3300R.U.* 3 x week	11,200 R.U. daily	- - - - -
	S.G.F. DOSE	- - - - -	- - - - -	- - - - -	- - - - -	15,150 R.U. 3 x week
	Thyroid DOSE	- - - - -	2 grs. daily	1 gr. daily	1 gr. b.i.d.	100 U. Thyrotropin 3 x wk.
6 Mos.	G.F. DOSE	- - - - -	- - - - -	3300 R.U.* 3 x week	14,000 R.U. daily	- - - - -
	S.G.F. DOSE	9600 R.U. 4cc. 3 x week	19,500 R.U. daily-3 x wk.	- - - - -	- - - - -	25,100 R.U. daily
	Thyroid DOSE	- - - - -	2 grs. daily	1 gr. daily	50 U. Thyrotropin daily	50 U. Thyrotropin daily
9 Mos.	G.F. DOSE	- - - - -	- - - - -	17,400 R.U. 3 x week	10,000 R.U. 3 x week	
	S.G.F. DOSE	13,000 R.U.	13,700 R.U. 3 x week	- - - - -	- - - - -	
	Thyroid DOSE	½ gr. t.i.d.	1 gr. t.i.d.	1 gr. daily	50 U. Thyrotropin 3x wk.	
12 Mos.	G.F. DOSE	- - - - -	- - - - -	15,600 R.U. daily		
	S.G.F. DOSE	18,000 R.U.	16,900 R.U.	- - - - -		
	Thyroid DOSE	½ gr. t.i.d.	1 gr. t.i.d.	1 gr. daily		
15 Mos.	G.F. DOSE	28,800 R.U.	- - - - -	21,600 R.U.		
	S.G.F. DOSE	- - - - -	20,000 R.U.	- - - - -		
	Thyroid DOSE	½ gr. daily	1 gr. t.i.d.	1 gr. daily		
REMARKS: G.F. = Growth Factor S.G.F. = Special Growth Factor; see text.		2 wk. rest period after every 4 wks. therapy. Dose increased gradually from 0.5-4 cc.	Treated 14 months.	No rest periods	Thyroid during rest periods. 8 mos. therapy	Treated 5½ mos. with rest periods.
		*Antuitrin-G employed				

FIGURE 4
TREATMENT SCHEDULE OF ALL CASES

expected growth of 1¾ inches, or 220 per cent increase over the expected normal increment. Even when the initial height was compared with a child of comparable height—i.e., an 11½ year old in whom the growth rate is greater than at 14½ years, the growth increment for 18 months still exceeded this by 0.8 inch (Figure 5). The accumulated growth was greater, by three inches, than that of a normal child of similar age on the 50 per cent curve, (Figures 6*A* and 6*B*).

The most rapid increases in growth was the one inch which occurred after the first three months of therapy. During the subsequent months of therapy there was a progressive decrease in the growth increment during each three month interval, even though the dosage of growth factor was increased, and thyroid added. During the 18 months of therapy she gained 20 pounds in weight, with an average of 3¾ pounds in each three month interval of therapy, or five pounds for each added inch in height. Her anthropometric measurements approached normal at a rate considerably greater than would otherwise have taken place

Case 1. Age 10.5 years	At Start	3 Mos.	6 Mos.	9 Mos.	12 Mos.	18 Mos.
Accomplished Height (inches)	53.0	53.6	54.2	55.0	55.7	57.0
Contemplated Height (inches) if untreated	53.0	53.4	53.6	53.9	54.2	54.75
Height for Age (inches) - 50% Curve (Burgess)	61.6	61.9	62.1	62.25	62.4	62.6
Normal Child of Comparable Height (10 years)	53.0	53.4	54.0	54.6	55.2	56.2
Increment Based on Contemplated Growth (inches)	0.0	0.4	0.6	0.9	1.2	1.75
Increment in Excess of Contemplated Growth (inches)	0.0	0.6	1.15	1.5	1.8	2.85
Actual Growth Increment (total)	0.0	1.0	1.75	2.4	3.0	4.0
Growth Increment of Child on 50% Curve (Burgess)	0.0	0.3	0.5	0.65	0.8	1.0
Actual Increment in Excess of Child on 50% Curve (Burgess)	0.0	0.7	1.25	1.75	2.2	3.0
Growth Increment of Child of Comparable Height	0.0	0.4	1.0	1.6	2.2	3.2
Actual Increment in Excess of Child of Comparable Height	0.0	0.6	0.75	0.8	0.8	0.8
Percentage Increase Over Contemplated Growth	0.0	150.0%	191.6%	166.6%	150.0%	129.0%
Weight (pounds)	60.0	63.75	67.0	71.25	73.5	80.0
Unit Dosage of Growth Factor	0	6,950	15,350	18,350	36,350	65,150

FIGURE 5

CASE 1: COMPARISONS IN HEIGHT AND GROWTH INCREMENTS AT VARIOUS INTERVALS DURING TREATMENT

(Figure 3). The growth was greatest in the long bones. Two and one-half inches of the four inches of total growth were in the lower measurement, and only 1½ inches in the upper measurement. From the staturogram it can be seen, by measuring the distance between parallel lines *B* and *A*, which represent 18 months of normal development, and the distance between *b* and *a*, which represents the rate of development during treatment, that the latter was greatly accelerated over these normal standards. (It must be realized that this child would probably develop at a rate much slower than normal, so that these differences are even greater than is apparent from this chart.) In addition to these anthropometric changes there was a concurrent advancement in the psychic behavior and social adjustment of the patient. During the period of therapy the patient's health was better than previously. As evidence that the growth preparation did not

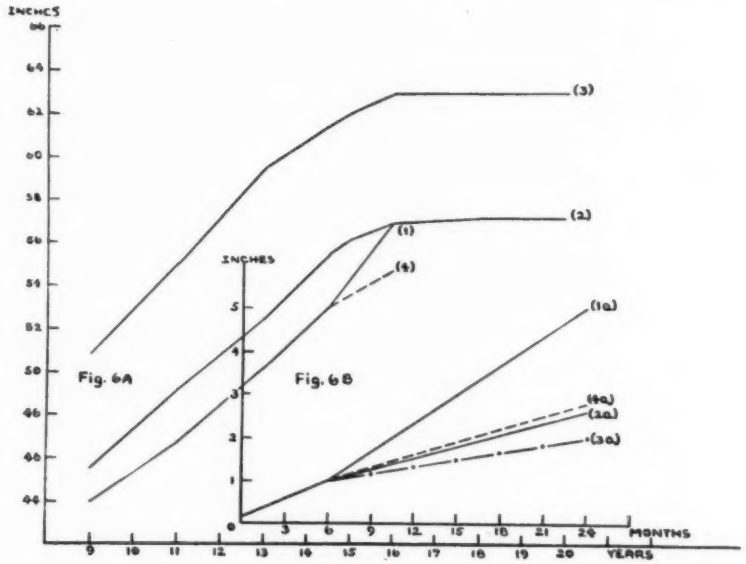

FIGURE 6

A. Mean Growth of Case 1 (1), Compared with Girls of Similar Age on 1 per cent Curve (2), on 50 per cent Curve (3), and the Contemplated Height of Patient if Untreated (4)

B. Comparison of Actual Growth Increment of Case 1 (1a), with that of Girl on 1 per cent Curve (2a), on 50 per cent Curve (3a), and Contemplated Increment of Patient if Untreated (4a)

exert any inhibitory action on either the pituitary or the ovary, it should be noted that after one year of therapy, menses were established and maintained at a cyclic intervals with a normal flow and little pain.

2. *Case 2*

J. K. was a 15½ year old male weighing 122 pounds, and 55¾ inches tall when first seen. At five years of age he had a severe case of measles following which there was an excessive gain in weight. The appetite was very good and polydypsia and polyuria were present. He had a high pitched voice and small larynx. During the six months prior to treatment there was beginning development of libido and secondary sex characteristics. The *IQ* was normal. The results of physical examination and laboratory studies suggested a diagnosis of hypo-

pituitarism resembling Fröhlich's syndrome. The appearance was that of a 12 year old (Figure 7). The lower body measurement exceeded

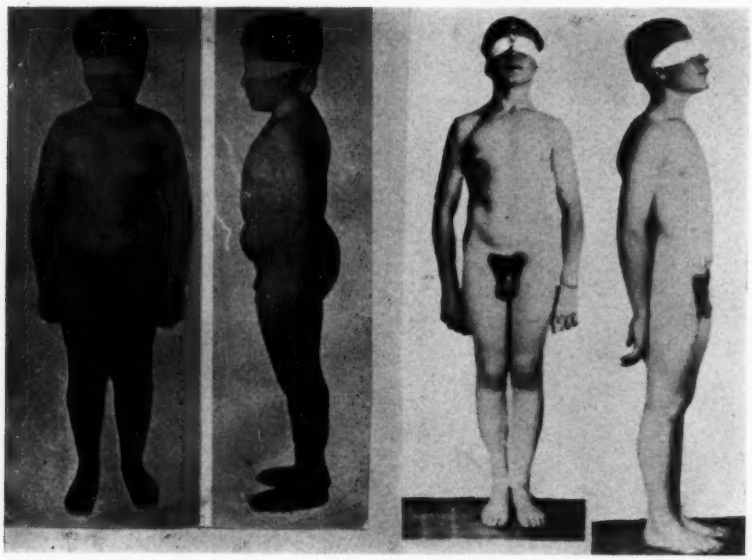

FIGURE 7

CASE 2 BEFORE (15½ YEARS) AND AFTER 14 MONTHS OF GROWTH THERAPY (16½ YEARS)

the upper and the span exceeded the height (Figure 8). The bone age corresponded to that of a boy of 13 years and the epiphyses were not fused.

Treatment was instituted at the age of 15½ years and G. F. was administered three times weekly (Figure 4). After five months the epiphyses were *x*-rayed and were not fused. Because of the adiposity the patient was placed on a subcaloric diet with adequate vitamin, mineral, and protein intake.

A growth of 4½ inches in 14 months, as compared with an expected growth of 0.85 inches in the same period, took place. Other comparisons reveal similar acceleration (Figure 9). The percentage increase in increment over the expected attainment was 429 per cent. The greatest period of growth was 3.37 inches during the first six months of treatment (Figure 10*A* and *B*), as compared with 1.15 inches during

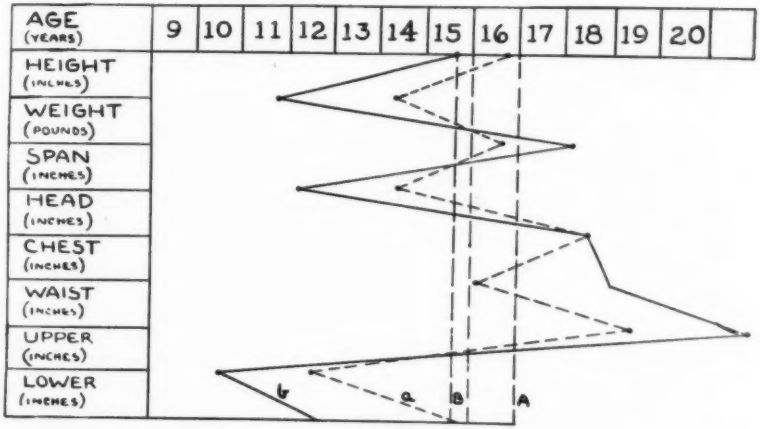

FIGURE 8

CASE 2: STATUROGRAM INDICATING NORMAL DEVELOPMENT (*A* TO *B*) AS COMPARED TO ACTUAL DEVELOPMENT (*a* TO *b*) UNDER GROWTH THERAPY

the last 8 months of therapy. This was probably due to the beginning of epiphyseal closure of the long bones in the latter period. A weight loss of 12½ pounds took place during an interval of 14 months during which there was a growth of 4½ inches, so that the weight reduction was much more significant than apparent from the actual figures. According to the staturogram (Figure 8) 2½ years of growth was accomplished in 14 months, and other measurements approached normality. Approximately 3 inches of the total growth of 4½ inches was accounted for by an increase in the size of the long bones. At the termination of therapy the appearance was that of a normally built boy of 16½ years, although somewhat short in stature (Figure 7). The childlike appearance which was present at the inception of treatment was replaced by that of a mature young adult and there was a concomitant maturation in mental outlook. There was no delay in puberty, as the latter had occurred during the course of treatment. There was a complete normal social adjustment. During the entire course of therapy there was no impairment of health. There was no apparent disturbance of the glandular physiology, as the testes and penis were that of a normal, well-developed youth and there was normal libido and sex function. The semen examination at the termination of treatment was normal.

Case 2. Age 15.5 years	At Start	3 Mos.	6 Mos.	9 Mos.	14 Mos.
Accomplished Height (inches)	55.75	57.25	58.15	59.6	60.25
Contemplated Height (inches) if Untreated	55.75	56.0	56.2	56.3	56.6
Height for Age (inches) - 50% Curve (Burgess)	64.6	65.1	65.6	66.0	66.4
Normal Child of Comparable Height (11.5 years)	55.75	56.2	56.6	57.2	57.9
Increment Based on Contemplated Growth (inches)	0.0	0.25	0.45	0.55	0.85
Increment in Excess of Contemplated Growth (inches)	0.0	1.25	2.92	3.38	3.65
Actual Growth Increment (total)	0.0	1.50	3.37	3.93	4.50
Growth Increment of Child on 50% Curve (Burgess)	0.0	0.5	1.0	1.4	1.8
Actual Increment in Excess of Child on 50% Curve (Burgess)	0.0	1.0	2.37	2.53	2.7
Growth Increment of Child of Comparable Height	0.0	0.45	0.85	1.45	2.15
Actual Increment in Excess of Child of Comparable Height	0.0	1.05	2.52	2.48	2.35
Percentage Increase Over Contemplated Growth	0.0	500.0%	648.0%	614.0%	429.0%
Weight (pounds)	122.0	121.0	117.0	113.0	109.5
Unit Dosage of Growth Factor	0	15,250	20,425	39,925	53,625

FIGURE 9

CASE 2: COMPARISONS IN HEIGHT AND GROWTH INCREMENTS AT VARIOUS INTERVALS DURING TREATMENT

3. *Case 3*

S. B. was a 15 year old male weighing 113 pounds, and 56½ inches tall on his first visit. At 13 years of age there had been a rapid gain of weight which was distributed mainly about the pelvic girdle. He avoided the company of boys of his age because of his short stature and lack of proficiency in athletics.

Examination revealed an obese white male with feminine configuration and a short stature corresponding to a 12 year old child resembling Case 2. The lower body measurements exceeded the upper and the span exceeded the height (Figure 11). The epiphyses were open and the bone age corresponded to that of a boy of 12 years. A diagnosis of hypopituitarism of the Fröhlich type was made.

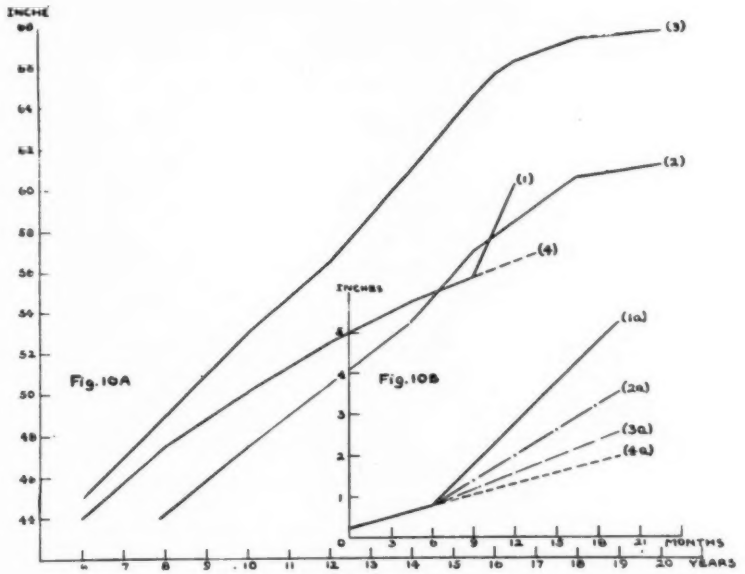

FIGURE 10

A. Mean Growth Curve of Case 2 (1), Compared with Boys of Similar Age on 1 per cent Curve (2), on 50 per cent Curve (3) and also with Contemplated Growth of Patient if Untreated (4)

B. Comparison of Actual Growth Increment of Case 2 (1a), with that of Boys on 1 per cent Curve (2a), on 50 per cent Curve (3a), and Contemplated Increment of Patient if Untreated (4a)

He was treated initially by another physician with Antuitrin-G (Figure 4). No anthropometric measurements had been made prior to treatment. His height prior to therapy was 56½ inches. During the first nine months period he grew an additional 2½ inches (Figure 12*A* and *B*). His actual increment for 18 months was 5½ inches as compared to his expected of 1¾ inches. His previous growth curve tended to follow the 10 per cent curve of Burgess. At 16 years his height of 62 inches was equivalent to that of a boy of similar age on the 15 per cent curve, indicating an appreciable acceleration in growth (Figure 12B). During the last 12 months of therapy his growth was almost equivalent to two years' growth. There was a redistribution of fat (Figure 11). There was a concomitant social adjustment and sexual maturation equivalent to that observed in the previous case.

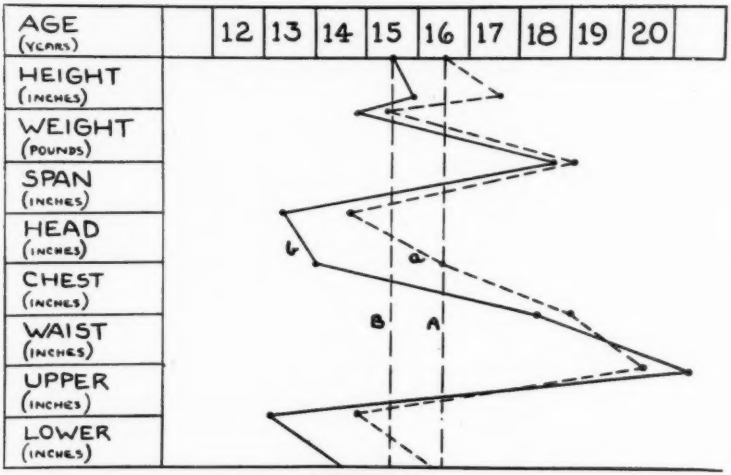

FIGURE 11

CASE 3: STATUROGRAM INDICATING NORMAL DEVELOPMENT (*A* TO *B*) AS COMPARED TO ACTUAL DEVELOPMENT (*a* TO *b*) UNDER GROWTH THERAPY

4. *Case 4*

S. D. was a 6½ year old female weighing 35¼ pounds, and measuring 35¼ inches in height on her initial visit. She was a full-term baby, delivered spontaneously, and weighed 8 pounds and 6 ounces at birth. At nine weeks of age, when development was retarded, an *x*-ray diagnosis of chondrodystrophy was made. Subsequent growth was very slow. Other than the physical deformity, the remaining childhood history was not significant. There was a younger sister who was developing normally. The parents were normal and there was no familial history of chondrodystrophy. The *IQ* of the patient was above normal.

Examination revealed an alert female child with the bodily configuration and physiognomy typical of achondroplasia (Figure 13). Her height corresponded to a child of 2½ years. The patient's height fell below the 1:10,000 curve (Figure 14). Laboratory studies were normal except for a blood cholesterol of 283 mg. per cent. *X*-ray showed the extremities of the bones to be short and flat. Epiphyseal development was normal for her age. The sella was normal.

During the 8 months of treatment, she grew 1¾ inches, as compared with an expected 0.2 inches based on previous growth rate (Figure 15). A normal child of the same age on the 50 per cent curve of Burgess

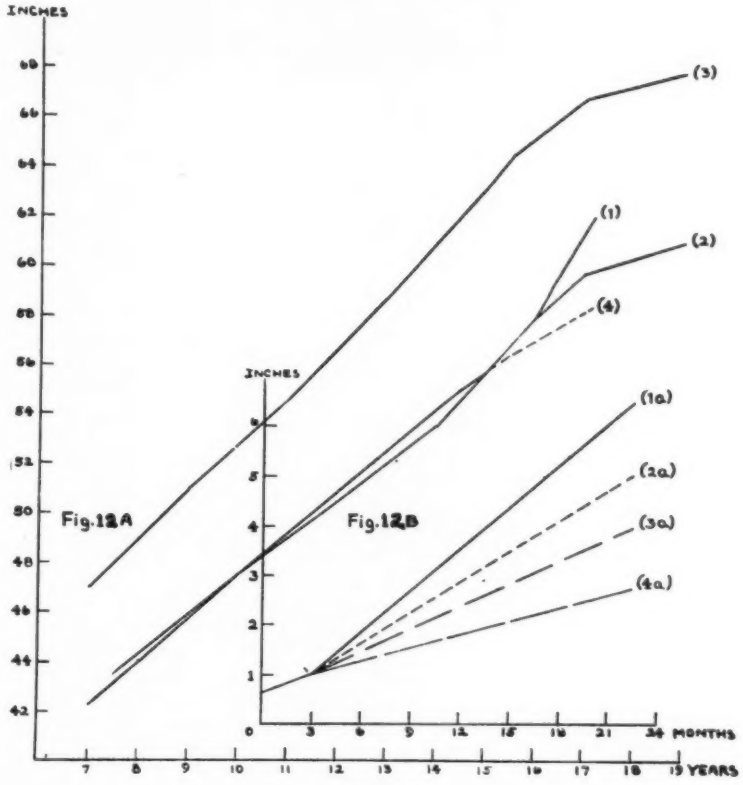

FIGURE 12

A. Mean Growth Curve of Case 3 (1), Compared with Boys of Similar Age on 1 per cent Curve (2), on 50 per cent Curve (3), and Expected Height of Patient if Untreated (4)

B. Comparison of Actual Growth Increment of Case 3 (1a), with that of Boy on 1 per cent Curve (2a), on 50 per cent Curve (3a), and Contemplated Increment of Patient if Untreated (4a)

would show an increment of 1.4 inches during the same period (Figure 14). As this patient was an achondroplastic, there were no figures available as to expected growth and it was necessary to utilize her previous rate of growth as a guide in anticipating her probable expected gain in height. The greatest stimulation was accomplished in the first three months of treatment. During this time she grew one

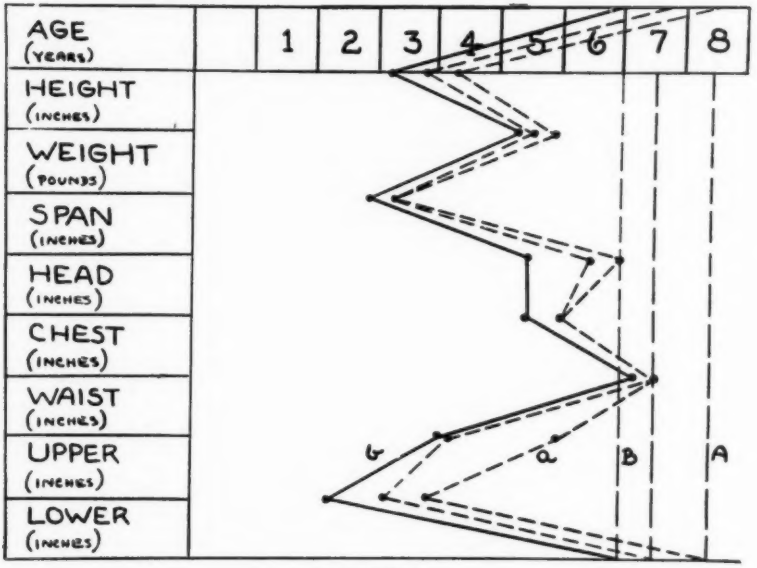

FIGURE 13

CASE 4: STATUROGRAM INDICATING NORMAL DEVELOPMENT (*A* TO *B*) AS COMPARED TO ACTUAL DEVELOPMENT (*a* TO *b*) UNDER GROWTH THERAPY

inch. This was substantially greater than either a child of similar age on the 50 per cent curve, or her own anticipated gain without treatment and was slightly better than a child of similar age on the 1:10,000 curve (Figure 14*B*). There was a marked increase in lower measurement as compared to upper, the lower being equivalent to almost a year's growth (1.55 inches) and the upper negligible (0.2 inches) (Figure 13).

This case was followed for one year after cessation of treatment. During this time she grew an additional two inches as compared with 0.3 inches on contemplated growth based on the previous rate. Her total growth for 20 months was 3¾ inches as compared to ½ inch of expected growth, 3½ inches for the 50 per cent curve, and 3.6 inches for the 1:10,000 curve. The growth rate for this period of time exceeded that obtained in each of the other standards. This was equivalent to an increase of 605 per cent as compared with her expected gain. From 6½ years to 8 years of age (Figure 14) the patient mani-

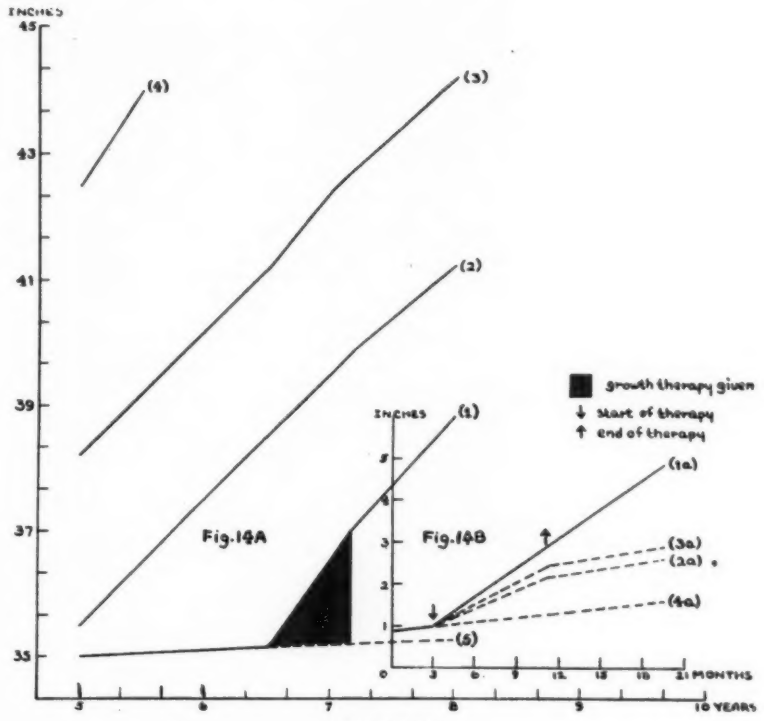

FIGURE 14

A. Mean Growth Curve of Case 4 (1), Compared with Girls of Similar Age on the 1:10,000 Curve (2), on the 1 per cent Curve (3), on the 50 per cent Curve (4), and Expected Growth if Untreated (5)

B. Comparison of Actual Growth Increment of Case 4 (1a), with that of Girls on the 1:10,000 Curve (2a), on the 1 per cent and 50 per cent Curve (3a), and Contemplated Increment of Patient if Untreated (4a)

fested slightly more than one year's normal growth. The only other significant change in anthropometric measurements was an increase of 1.4 inches in the upper measurement. This was equivalent to slightly less than two years as compared with 2.35 inches for the lower measurement, which was equivalent to slightly over 1½ years. This was far more important than seemed apparent, for in these cases the growth of the long bones is markedly diminished. Furthermore, the growth of the lower extremities was greater (1.55 inches in 8 months) than that

Case 4. Age 6.5 years	At Start	3 Mos.	8 Mos.	14 Mos.	19 Mos.
Accomplished Height (inches)	35.25	36.25	37.0	38.2	39.0
Contemplated Height (inches) if untreated	35.25	35.35	35.45	35.65	35.75
Height for Age (inches) - 50% Curve (Burgess)	45.6	46.0	47.0	48.0	49.1
Normal Child of Comparable Height (2.25 years)	38.1	39.0	40.3	41.1	41.7
Increment Based on Contemplated Growth (inches)	0.0	0.1	0.2	0.4	0.5
Increment in Excess of Contemplated Growth (inches)	0.0	0.9	1.55	2.55	3.25
Actual Growth Increment (total)	0.0	1.0	1.75	2.95	3.75
Growth Increment of Child on 50% Curve (Burgess)	0.0	0.4	1.4	2.4	3.5
Actual Increment in Excess of Child on 50% Curve (Burgess)	0.0	0.6	0.35	0.55	0.25
Growth Increment of Child of Comparable Height	0.0	0.9	2.2	3.0	3.6
Actual Increment in Excess of Child of Comparable Height	0.0	0.1	-0.45	-0.05	0.15
Percentage Increase Over Contemplated Growth	0.0	900.0%	777.0%	637.0%	605.0%
Weight (pounds)	35.25	36.0	37.0	40.0	42.0
Unit Dosage of Growth Factor	0	17,200	35,200	- -	- -

FIGURE 15

CASE 4: COMPARISON IN HEIGHT AND GROWTH INCREMENTS AT VARIOUS INTERVALS DURING TREATMENT

which took place during the subsequent untreated period (0.8 inches in 11 months). Early in therapy there was an apparent alteration in psychic behaviour from that of a child less than her age to that of an older child. Irritability, independence, and lack of discipline were also present. The intensive therapy did not impair her general health. Her appearance was somewhat changed (Figure 16).

5. *Case 5*

S. B. was a three year old male, weighing 26 pounds and 29¼ inches tall when first seen. He was the first-born of twins and was delivered by forceps. The birth weight was 6½ pounds. Development was moderately retarded. The child was brighter than his twin brother who was normally developed. The family history was negative.

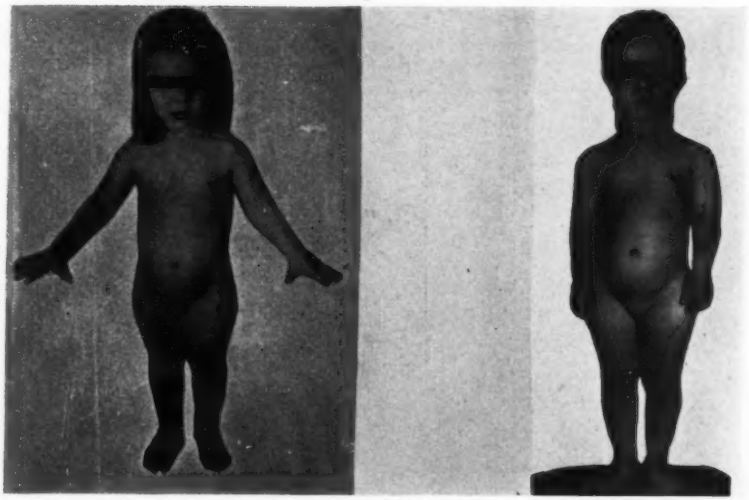

FIGURE 16
CASE 4: SHOWING COMPARISON BEFORE (6½ YEARS) AND AFTER (8 YEARS) GROWTH THERAPY

Examination showed a bodily configuration similar to that of the previous case and a height corresponding to that of a child of one year (Figure 17). According to the Burgess chart the patient's height was below the 1:10,000 curve (Figure 18).

Treatment consisted of S. G. F. and thyrotropic given for only five and one-half months because of a paucity of material. He was also given ultra-violet irradiation three times a week (Figure 4). Comparison with expected growth could not be made because previous growth data were not available. A comparison with a child of similar height would not be significant because in a normal child of less than one year the growth is very rapid. Subsequent to a five-month period of therapy in which there was a growth of 1¾ inches, the patient was observed for an additional seven months. During this time he grew 1.55 inches, making a total of 3.3 inches as compared with 2.6 inches on the Burgess 50 per cent curve and 2.3 inches on the 1:10,000 curve (Figure 18). From the third to the fourth month of treatment the growth was equivalent to more than one year's normal growth (Figure 17). The upper measurements revealed an acceleration of more than

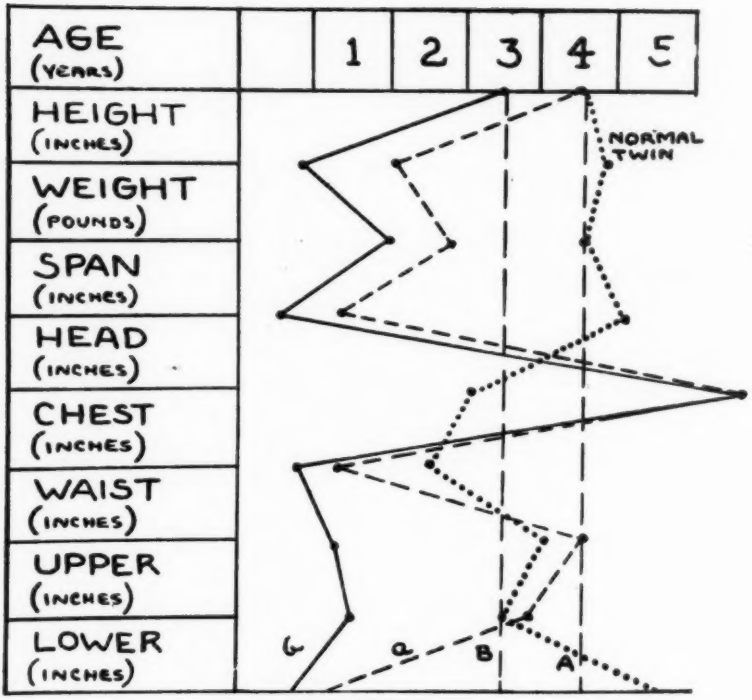

FIGURE 17

CASE 5: STATUROGRAM INDICATING NORMAL DEVELOPMENT (*A* TO *B*) AS COMPARED TO ACTUAL DEVELOPMENT (*a* TO *b*) UNDER GROWTH THERAPY, AND COMPARED TO NORMAL TWIN (DOTTED LINE)

two years' normal growth, whereas the growth in the long bones was only equivalent to one-half year of normal growth. A comparison with the normal twin may be made with facility from staturogram. The greatest growth was during the first five months of treatment (Figure 19). It appeared that there was a change in facies with less bossing after this 12-month period and there was a slight straightening of the limbs (Figure 20). The child enjoyed good health during treatment.

DISCUSSION

Intensive therapy consisting of large and frequent doses of anterior pituitary growth extract was instituted in these cases in order to stimu-

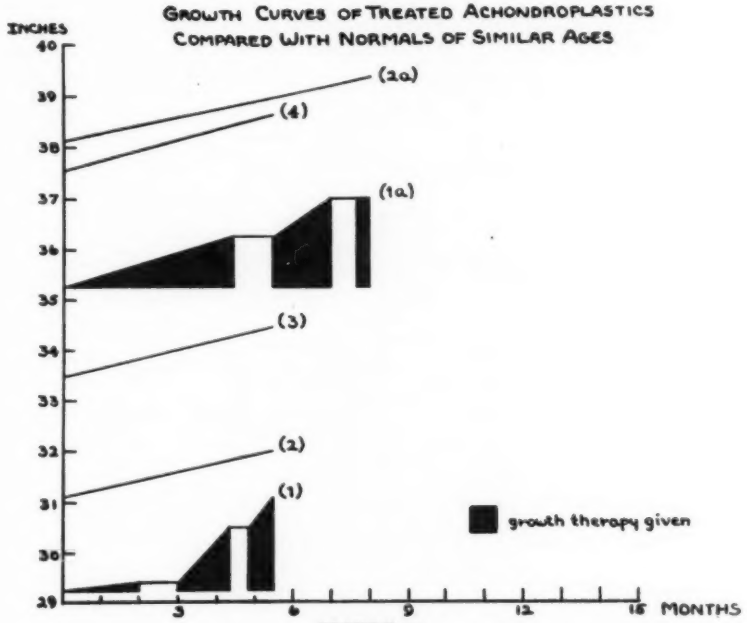

FIGURE 18

ACTUAL GROWTH CURVE OF CASE 5 (1), COMPARED WITH BOYS OF SIMILAR AGE ON 1:10,000 CURVE (2), 1 PER CENT CURVE (3), AND 50 PER CENT CURVE (4); AND THE ACTUAL GROWTH OF CASE 4 (1a), COMPARED WITH GIRLS OF SIMILAR AGE ON THE 1:1,000 CURVE (2a)

late the growth of the long bones. It has been shown by Selye (23), and Freud *et al.* (14), that the site of action of the growth factor of the pituitary is principally on the cartilaginous centers.

A staturogram based on age-measurement relationship devised by Sharp (24) was utilized in studying these cases. The normal averages of height, weight, span, head, chest, waist, upper and lower measurements compiled by Engelbach (10) were tabulated in vertical columns for each age group (Figure 1). The figures for the female are in the upper half of the block and for the male in the lower half. Hence, by reading straight down through a column one can determine the ideal normal dimensions for a child at a given age. It is of course rare to obtain such a straight vertical line, however a straight line trend will indicate measurements within the normal range. The actual measure-

Case 5. Age 3 years	At Start	2 Mos.	5 Mos.	9 Mos.	12 Mos.
Accomplished Height (inches)	29.2	29.38	30.95	31.65	32.5
Height for Age (inches)— 50% Curve (Burgess)	37.6	38.1	38.9	39.5	40.2
Child Same Age on the 1:10,000 Curve (Burgess)—(inches)	31.1	31.4	32.0	32.6	33.4
Actual Growth Increment (total inches)	0.0	0.18	1.75	2.45	3.3
Increment of Child Same Age on 50% Curve (inches)	0.0	0.5	1.3	1.9	2.6
Increment of Child Same Age on 1:10,000 Curve (inches)	0.0	0.3	0.9	1.5	2.2
Actual Increment in Excess of Child on 50% Curve (inches)	0.0	—0.32	0.45	0.55	0.7
Actual Increment in Excess of Child on 1:10,000 Curve (inches)	0.0	—0.12	1.85	0.95	1.0
Weight (pounds)	24.25	25.25	26.5	27.3	28.0
Unit Dosage of Growth Factor	0	15.150	41,600	– –	– –

FIGURE 19

CASE 5: COMPARISONS IN HEIGHT AND GROWTH INCREMENTS AT VARIOUS INTERVALS DURING TREATMENT

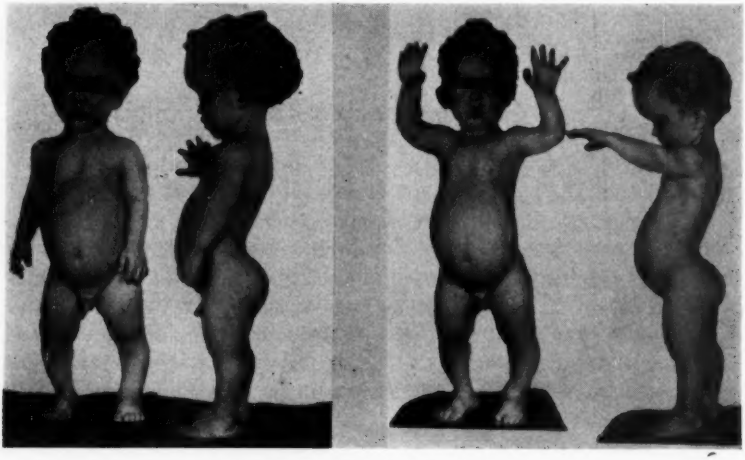

FIGURE 20

CASE 5: BEFORE (3 YEARS) AND AFTER (4 YEARS) THERAPY WITH GROWTH EXTRACT

ments of the patient were plotted prior to treatment and at subsequent intervals during therapy. It was found in the hypopituitary dwarf who resembled a Lorain-Levi type, that all measurements deviated sharply to the left, toward the lower age brackets. In the obese hypopituitary dwarf who resembled a Fröhlich (Case 2, age 15½ years), the height, span, upper and lower measurements deviated to the right toward the higher age bracket.

In the achondroplastics, there was a sharp deviation to the left in height, span, and upper and lower measurements, while the circumference of the head and waist tended to be within normal limits. This staturogram served as an excellent graphic guide in recognizing, at a glance the response to therapy. A measurement of the distance between the age at the start of treatment and the age at any point during treatment, when applied to each measurement, taken at comparable times, revealed the somatic response in age-years to therapy. For example, the linear distance equivalent to one year when compared with the distance between successive similar measurements indicated the developmental progress in terms of time as well as of measurement.

In carrying out treatment with the growth preparations, it is preferable to institute therapy at as early an age as possible in order to allow a greater span for treatment before epiphyseal union. Moreover, if there was sexual retardation, adequate therapy in correcting this could be given after maximum growth responses had taken place. Treatment for undescended testes is best instituted before epiphyseal union. Furthermore, in young children, the dietary growth response to high-caloric, high-vitamin intake is much greater than in older children.

No extensive metabolic studies were carried out in these patients before or after therapy. The blood calcium and inorganic phosphorous showed very little variation while the lipoid phosphorous and cholesterol were reduced in Cases 1 and 2. There were no alterations in the hematological picture and the urinalyses were always normal. Glycosuria did not occur.

Treatment was instituted with small doses which were gradually increased to the optimal dose, which averages 3 cc. This differed from the therapy outlined by other investigators principally in the fact that a higher dose was administered more frequently and over a greater period of time.

The writers had anticipated that the time of epiphyseal union would be delayed beyond the normal expectancy. This was not the case, for

x-ray studies of the bones in the first two cases indicated that there was an acceleration in bony development, ossification, and epiphyseal union. In these two cases, it is possible that the acceleration of epiphyseal union was attributable to some extent to the genital maturation. It is possible that the advance in epiphyseal union was unaffected by the growth therapy per se but was taking place at a predetermined rate. If epiphyseal union was taking place in these two cases because of the inhibition of the gonadal hormones on the pituitary, the growth accomplished was even greater than the normal expectancy. The fact remains, however, that in the first two cases which were treated for approximately 18 months, there was an acceleration in ossification and epiphyseal union of approximately three years. How much of this was spontaneous and how much was attributable to the therapy cannot be definitely stated. It is hoped that this fact will be clarified eventually by observation of an increased number of cases.

The thyroid and thyrotropic substances administered to these patients may have contributed to the growth responses and the bony changes observed. The diet, high in caloric, protein and vitamin content furnished the necessary supply of material for the newly formed tissue. It is possible that the amino acids, essential for new growth, augmented the action of the growth factor. These constitute an important factor in the production of new growth (16, 17) and should be given careful attention by the therapeutist. They become doubly important in cases where accomplishment of excessive growth stimulation is attempted. Every facility was utilized to stimulate the appetite in these patients, and extra feedings were employed.

When treatment was instituted in Patient 1 her growth curve fell on about the 1:5000 curve of Burgess (Figure 6). There was almost an immediate growth response to therapy and the curve rose gradually until it intersected the 1 per cent curve at 18 months. In other words, at the inception of treatment, 4,999 children per 5000 of similar age were taller than she, while at the termination of therapy only 99 out of 100 were taller. It is the opinion of the authors, as well as others (Lurie and Dorff), that the greater the retardation in height, the poorer the response to growth therapy. Judging by previous growth in Case 1, it appeared likely that, without the treatment, the expected growth might not have exceeded by much the 1:5000 curve of Burgess (Figure 21).

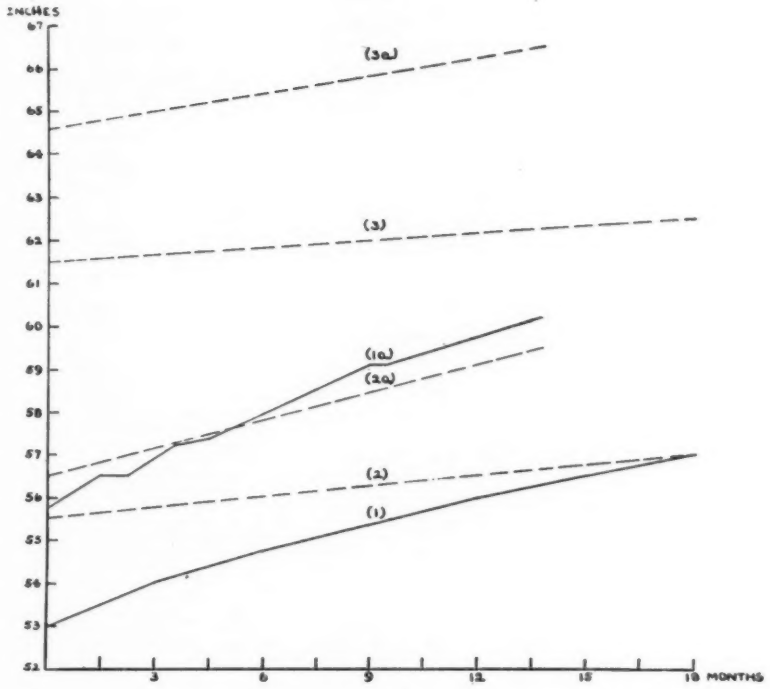

FIGURE 21

A Comparison of the Actual Growth Curve of Case 1 (1), with that of a Girl on 1 per cent Curve (2), and 50 per cent Curve (3); and a Comparison of the Actual Growth Curve of Case 2 (1a), with that of a Boy on 1 per cent Curve (2a), and 50 per cent Curve (3a)

In Case 2 the initial growth curve fell on the 1:2000 curve of Burgess. By the fourth or fifth month of treatment growth was accelerated to the extent that the curve intersected the 1 per cent curve (Figure 21). The growth spurt continued until at the end of 15 months of therapy he was on the 3 per cent curve. It is probable that in Case 2, if treatment had not been given, the growth would not have exceeded the 1:2000 curve (Figure 10). This served to indicate that gratifying results rewarded the therapy in these cases.

Burgess found that in girls, maximum growth was reached by the 13th year and gradually leveled off to a plateau at 17 years, while in

boys this took place at 15 years and 21 years. Therefore, it would be less likely for any spontaneous growth, to the extent seen in these two cases, to have taken place. The increase in body weight in Case 1 exceeded even the rate of increase in height, for there was a gain of 20 pounds in 18 months which was equivalent to a weight gain of 27 months. The staturogram (Figure 3) indicated that there was an alteration in configuration from the infantile to the more mature type. On the other hand, in Case 2, a weight loss of 13 pounds was observed in 15 months, which brought the weight within normal limits for the age.

In Case 3 during the first six months of therapy there was an acceleration of 336 per cent over the contemplated growth, based on previous records (Figure 22). At the termination of 18 months of therapy the

Case 3. Age 15 years	At Start	3 Mos.	6 Mos.	9 Mos.	12 Mos.	18 Mos.
Accomplished Height (inches)	56.5	57.5	58.1	59.0	59.5	62.0
Contemplated Height (inches) If untreated	56.5	56.75	57.1	57.3	57.6	58.25
Height for Age (inches) - 50% Curve (Burgess)	64.6	65.1	65.6	66.0	66.2	66.7
Normal Child of Comparable Height (11.4 years)	55.5	55.8	56.2	56.7	57.2	58.3
Increment based on Contemplated Growth (inches)	0.0	0.25	0.6	0.8	1.1	1.75
Increment in Excess of Contemplated Growth (inches)	0.0	1.0	2.02	2.7	2.9	3.75
Actual Growth Increment (total)	0.0	1.25	2.62	3.5	4.0	5.5
Growth Increment of Child on 50% Curve (Burgess)	0.0	0.5	1.0	1.4	1.6	2.1
Actual Increment in Excess of Child on 50% Curve (Burgess)	0.0	1.0	1.62	2.1	2.4	3.4
Growth Increment of Child of Comparable Height	0.0	0.3	0.7	1.2	1.7	2.8
Actual Increment in Excess of Child of Comparable Height	0.0	0.95	1.92	2.3	2.3	2.7
Percentage Increase Over Contemplated Growth	0.0	400.0%	336.0%	337.0%	262.0%	214.0%
Weight (pounds)	113.0	107.5	115.0	123.0	124.5	128.5
Unit Dosage of Growth Factor	0	6,600	15,300	24,000	31,800	53,400

FIGURE 22

CASE 3: COMPARISONS IN HEIGHT AND GROWTH INCREMENTS AT VARIOUS INTERVALS DURING TREATMENT

percentage increase over the calculated increment was 214 per cent. Here again bodily configuration and psyche even exceeded that of growth attainment.

In achondroplasia there seems to be such a disparity in the literature on the rate of epiphyseal union and ossification that no standards could be set up which would make a suitable comparison feasible. The individual variation was very wide. There are no statistics available on average growth rate in these cases. However, in six cases, whose records were available to us, all were retarded in height, and development was similar to those reported here. Also noted was a tendency to early epiphyseal union. It was learned however, that an occasional case with delayed epiphyseal union, due to the separation of the shaft from the epiphysis by the invasion of connective tissue from the periosteum has been encountered (29). The rate and degree of ossification in our cases proceeded normally. The authors have felt justified in attempting to stimulate growth in these cases because of the slow rate of growth. This was borne out by the results obtained in cases described.

In Case 4 most of the growth took place in the long bones as evidenced by the fact that the lower body measurement increased at a rate proportionately greater than did the upper. In view of the fact that in these cases the trunk measurements are usually normal, as most of the growth takes place here, it was felt that this effect on the lower body growth attests the activity of the product used. The beneficial change in appearance of this patient was greater than could be accounted for by the anthropometric measurements. *X*-ray studies subsequent to treatment revealed some straightening of the long bones of the legs, an increased deposition of calcium in the cortex of the bones, and considerable replacement of cartilaginous tissue by new bone.

In Case 5 there was a growth of 1¾ inches in a 5-month period of treatment. Here again, this was greater than a normal child of similar age on the 1:10,000 curve of Burgess. In this case, however, the greatest growth response occurred after the third month of treatment (Figure 18). Unlike the previous case most of the growth took place in the trunk, as the increase in upper body measurement was equivalent to more than two years of normal growth. Although growth may have been affected in the long bones, this was not apparent because of the continued bowing. According to *x*-ray the advancement in bone age was in keeping with the stimulated growth rate.

In none of the cases described herein were prolonged plateaus in growth observed, nor have any of the cases treated, including more recent ones not reported here, manifested any lack of response following the initial spurt on therapy. All of our cases continued to grow throughout the course of treatment.

According to most authors the epiphyses in cases of dwarfism, resulting from hypofunction of the pituitary gland, are usually delayed in closing. This was true of our cases. Hence one of the end results of the pituitary therapy was an advancement in bone age based on rapid union of the epiphyses and diaphyses. The period of observation in all of our cases has not yet terminated, so it is impossible to make statements concerning the ultimate height attained. However, measurements made at this interval of observation, reveal that Case 1 (A. C.) is 57 inches tall instead of a probable 54¾ inches; Case 2 (J. K.) is 60¼ inches tall instead of 56.6 inches; Case 3 (S. B.) is 62 inches tall instead of 58¼ inches; Case 4 (S. D.) is 39 inches tall instead of a probable 35¾ inches, and Case 5 (S. B.) is 32½ inches tall instead of a probable 30½ inches. This response in several of the cases meant a difference between being a definite dwarf and a short normal individual. We have no measurements on untreated achondroplastics.

The growth obtained in the hypopituitary dwarfs was proportionately distributed. The ratio of the upper to the lower body measurements was fairly well preserved throughout therapy.

Other therapeutic endocrine substances have been used to accomplish growth in retarded individuals. Outstanding among these substances was the recent employment of androgens. Observations have been made by Browne and Ross (2) on growth stimulation in hypogonadal individuals with testosterone propionate. They used dosages of 25 mg. twice weekly and were able to demonstrate an increase in bone age after varied periods of treatment. Others have reported on the effects of androgens on the skeletal growth of rats, claiming that small doses depress and large doses accelerate growth (21, 22). This accelerating action of androgen may be similar to that observed in cases of pubertas praecox as caused by pineal and adrenal cortex tumor. In a very tall child with adrenal cortex tumor whom we have studied, it was found that large quantities of androgens were excreted daily in the urine. However, in these cases, although there is a rapid growth spurt, it is of short duration and the ultimate height obtained is usually far below normal. This may occur in those cases treated with androgens,

as apparently, when the androgen level is sufficiently increased, there is depression of the pituitary with resulting closure of the epiphyses.

The authors have been inclined to look upon the results with androgens as an artificially produced "pubertal spurt." At the present writing we are not in agreement with those investigators who advocate the use of androgens in the treatment of dwarfism. It is felt that the approach in this type of therapy should be cautious as the long time results may prove to be less desirable than the existing condition. It is difficult to reconcile this androgenic stimulation of growth with the fact that in eunuchoid subjects, in whom the urinary titer of androgens is admittedly low, there is excessive growth. Dorff has employed gonadotropic extracts in the treatment of dwarfism and it is felt that the same evaluation is justifiable. It has been fairly well established that estrogens would be of no value in treating growth retardation, as work with animals by Zondek (31, 32) and others (15, 20) has shown convincingly that large doses of estrogens will inhibit the pituitary and cause dwarfism. In a series of cases of gonorrheal vaginitis in children varying in age from 2 to 14 years, treated with estrogen by one of us (F. R. S.), the effect on subsequent growth was irregular. Irrespective of dose, most cases, in which a follow-up could be obtained, grew at a normal rate. However, in one child in whom treatment was instituted at three years of age, there was a growth of only one inch in almost four years. It is planned to check this series further and subject it to statistical analysis so that a definite statement can be made. During the interim, this form of treatment should be instituted with due consideration to the possible ultimate untoward effect.

Subsequent cases of dwarfism, including both the hypopituitary and achondroplastic types, have been under treatment with various pituitary extracts. In this series an attempt is being made to more rigidly control and study the progress under therapy. In addition to various innovations in supportive therapy, we have employed two other sources of growth-promoting material. One, a concentrated preparation of anterior pituitary extract made according to the principles outlined by Collip, and two, a highly purified preparation of prolactin prepared by Dr. Schwenk of the Schering Corporation. The latter preparation is being utilized because of the possibility that the lactogenic principle of the anterior pituitary and the alleged growth hormone are identical. Although this latter preparation has been employed only a few months, growth responses have already been observed. These results will be presented in a subsequent publication.

Summary

1. Diagnostic criteria and growth therapy, in cases of hypopituitarism, pituitary infantilism and achondroplasia are outlined.

2. Treatment consisted of frequent and large doses of anterior pituitary growth extract, a special growth extract of the anterior pituitary, thyrotropic factor, thyroid, dicalcium phosphate and viosterol, thiamin chloride, essential amino acids and a high protein diet.

3. Curves indicating acceleration of growth along increased rate levels are depicted.

4. Alterations in anthropometric measurements and patterns are depicted in staturograms.

5. The actual growth accomplished in the five cases as compared to contemplated growth was:—in Case 1, 4 inches instead of 1¾ inches; in Case 2, 4½ inches instead of 0.9 inch; in Case 3, 5½ inches instead of 1¾ inches; in Case 4, 3¾ inches instead of 1 inch (achondroplasia) and in Case 5, 3 and 1/3 inches instead of 1 inch (achondroplasia).

6. Tentative results utilizing prolactin as a source of growth promotion are mentioned.

We are indebted to Drs. E. Schwenk and W. H. Stoner of the Schering Corporation for their generosity in supplying prolactin and thyroid extract. We particularly appreciate the constant encouragement and suggestions of Dr. William H. Stoner.

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